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PHOSPHORYLASE OF THE SKIN

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A number of papers [1-4] show that the skin is an organ in which the processes of carbohydrate, fat, protein and mineral metabolism take place at a fairly intensive rate. Wohlgemuth [5] established that the skin occupies a leading place with respect to the quantity of various enzymes.

According to the data of Kiverin [6], there is considerable amylase activity in the skin. However, there is another path for the decomposition of carbohydrates: phosphorolysis. In the present work we have posed the problem of clarifying the role and significance of the phosphorolytic system of enzymes in the decomposition of carbohydrates in the skin.

First it was necessary to establish the presence in the skin of phosphorylase.

METHOD

The subject of the investigation was an aqueous extract of the skin of the guinea pig. The crushed and ground skin was extracted in the cold for thirty minutes with two volumes of distilled water. One mg of this extract was incubated for 1 hour at 37°C with 1 ml of M/15 phosphate buffer, pH 6.8, containing 10 ml of glycogen, 0.5 ml of 0.25 M $MgCl_2$ and 0.4 ml 0.25 M NaF. The enzymatic processes were stopped after incubation by precipitating the proteins with an equal volume of 5% trichloracetic acid.

The sodium fluoride was added to inhibit the dephosphorylation of the glucose-phosphorus esters formed on the phosphorolysis of glycogen.

In the filtrate from the trichloracetic acid the inorganic and total phosphorus were determined by the Braunstein modification of the Fiske and Subbarow method. The content of reducing substances and the loss in glycogen were determined by Voinar's method.

EXPERIMENTAL RESULTS

TABLE 1

Phosphorylase Activity in Aqueous Extracts of Guinea Pig Skin.

Loss of inorganic phosphate (mg per sample)	Increase in organic phosphorus (mg per sample)	Loss of inorganic phosphate (mg per sample)	Increase in organic phosphorus (mg per sample)
0.004	–	–	0.018
0.024	–	0.008	0.004
0.021	–	0.013	0.020
0.045	0.033	0.044	0.047
0.004	–	0.023	0.044
0.004	0.048	0.016	0.020
–	0.065	0.018	–
0.036	0.055	0.020	0.036
0.008	0.026		

EXPERIMENTAL RESULTS

In Tables 1 and 2 are cited the results of the experiments in which the phosphorylase activity was studied.

Table 1 shows that on incubation there occurs an insignificant loss in inorganic phosphate and a correspondingly small increase in organic phosphorus compounds under the action of the skin phosphorylase.

TABLE 2

Reducing Power and Glycogenolysis in Aqueous Extracts of Guinea Pig Skin.

Increase in reducing substances (mg per sample)	Glycogen loss (mg per sample)	Increase in reducing substances (mg per sample)	Glycogen loss (mg per sample)
0.69	0.946	0.35	0.909
0.76	1.613	0.33	1.260
0.56	1.151	0.51	1.112
0.52	–	0.38	1.521
0.77	1.541		

The results of the experiments to determine the reducing power and glycogen loss given in Table 2 show that after incubation of the samples a considerable increase in the content of reducing substances occurs.

Comparison of Table 1 and Table 2 shows that the loss inorganic phosphorus does not correspond to the increase in reducing substances. The considerable formation of the latter may be the result not only of the subsequent action of phosphorylase and phosphatase on the substrate, but also of amylase.

The action of the phosphatase in our experiments was inhibited by sodium fluoride. To explain the participation of amylase in the process of formation of sugar, we studied the splitting of glycogen in the presence of systems which inactivate the amylase but have no effect on the phosphorylase and phosphatase.

TABLE 3

Change in Inorganic Phosphorus and Glycogen Content on Glycogenolysis of Aqueous Extracts of the Skin of Guinea Pigs.

Combining of inorganic phosphorus (mg. per sample)		Increase in reducing substances (mg. per sample)	
Without $HgCl_2$	With $HgCl_2$	Without $HgCl_2$	With $HgCl_2$
–	0.03	–	0.09
–	0.02	–	0.0
0.02	0.03	0.92	0.06
0.03	0.02	0.93	0.04
0.01	0.01	0.88	0.03

In the experiments we used 0.5 ml of a 3% solution of mercuric chloride per sample. From the results given in Table 3 it is seen that in the presence of mercuric chloride the increase in reducing substances is very small. This shows that most of the sugar is formed by the amylolytic activity of the extract.

Thus, determination of the combining rate of mineral phosphate and of the increase in reduction showed that there is phosphorylase in the skin, but that it is only very slightly active.

To answer the question of the possible ways of activation of skin phosphorylase, two series of experiments were carried out. In one, the effect of pH was studied. As can be seen from the results given in Table 4, change in pH between the limits 6.5 to 8.0 had little effect on the combining rate of inorganic phosphate.

In more acid (pH5.9) and more alkaline (pH 11) media the action of skin phosphorylase decreases considerably.

In the other series of experiments we studied the effect of irradiation with ultraviolet rays on the activity of skin phosphorylase.

Kaplansky [4] shows that under the influence of ultraviolet rays a considerable change in the carbohydrate metabolism of the skin takes place: during protracted exposure at high doses, an increase in sugar in the skin is observed. In our laboratory, an increase in the sugar of the skin following irradiation was also established; in this process the amylase activity decreased. Naturally the question arose: must not the increase in reduction following exposure be explained by an increase in the phosphorolytic activity of the skin?

TABLE 4

Decrease in the Phosphorus of Inorganic Phosphate Compounds on the Phosphorolysis of Glycogen (mg P per sample) as a Function of the Hydrogen Ion Concentration.

pH					
5.9	6.5	7.0	7.5	8.0	11.0
0.006	0.023	0.023	0.050	0.031	0.011
0.002	–	+0.020	0.004	0.030	+0.002
0.004	0.005	0.030	0.030	±0	+0.014
0.005	0.028	0.040	0.029	0.016	0.007
+0.002	–	0.004	0.012	+0.030	+0.01

The symbol(+)designates samples in which the quantity of inorganic phosphorus increased.

TABLE 5

Effect of Irradiation on the Combining rate of Inorganic Phosphate (mg per sample) with Aqueous Extracts of Guinea Pig Skin.

Exposed animals		Control animals (not exposed)	
Samples of skin before exposure	Samples of skin after exposure	First skin samples	Second skin samples
0.044	0.056	+0.012	+0.017
+ 0.068	0.058	0.010	0.006
0.006	0.030	0.008	±0.0
0.026	0.080	0.024	0.016
0.008	0.030	±0.0	+0.008
+ 0.03	0.038	–	–
0.048	0.064	–	–
0.013	0.061	–	–
0.016	0.043	–	–

In the experiment we used two groups of guinea pigs. One of the groups was exposed to a bactericidal lamp for 15 minutes; the other group was not exposed and served as controls. Skin for analysis was taken twice: before exposure (from one side) and after exposure (from the other). The phosphorylase activity was also determined twice in the controls. We wanted to exclude the effect of trauma by setting up control experiments.

The results of the experiments are given in Table 5; they indicate that after 1 hour of incubation of the mixture the phosphorus has combined at a higher rate in samples with extracts of skin taken after exposure. Thus phosphorylase activity of aqueous skin extracts exposed to ultraviolet rays is increased.

SUMMARY

The phosphorylase activity of aqueous extracts of skin is very low. The optimum pH lies within the range 5.9 to 8.0. Exposure of the skin to ultraviolet increases the phosphorylase activity of its extracts.

Received February 5, 1956

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SPECTROPHOTOMETRIC DETERMINATION OF DEHYDROGENASE ACTIVITY IN GREEN SUSPENSIONS

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Investigation of the activity of the dehydrogenase of green tissues by Thunberg's method is made more difficult because the color of the material interferes with observation of the decolorization of methylene blue.

Recently, wider use has been made of spectrophotometric methods of determining dehydrogenase in colorless tissues, based on the changes in color of various hydrogen acceptors (2,6-dichlorophenolindophenol, tetrazole salts, etc. [1]). The purpose of the present work was to arrive at a spectrophotometric method of determining the dehydrogenase activity in green suspensions. The use of methylene blue as a hydrogen acceptor did not seem favorable, since this dye has a characteristic absorption maximum in the same region as chlorophyll (about 600 mμ). Important factors in the choice of a dye were its absorption spectrum and also its oxidation-reduction potential. The absorption maximum of a hydrogen acceptor should lie in the region of least absorption by chlorophyll, while the magnitude of the oxidation-reduction potential E_0' should be close to that of methylene blue. These conditions are fulfilled by an analog of methylene blue, thionine, which has an E_0' at pH 7.0 of + 0.62 V (the E_0' of methylene blue at pH 7.0 is + + 0.05 V). The absorption spectra of thionine, methylene blue, and chlorophyll are shown in Fig. 1.

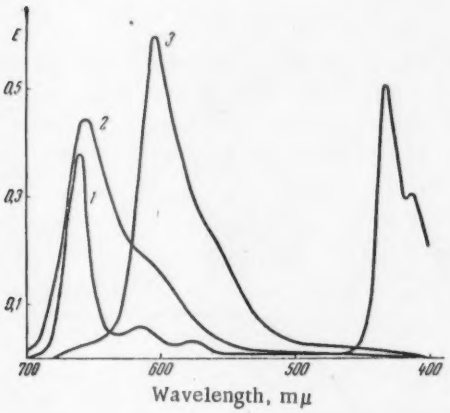

Fig. 1. Absorption spectra: 1) chlorophyll; 2) methylene blue; 3) thionine.

The kinetics of decolorization of thionine by green suspensions were measured initially on a Beckmann spectrophotometer at 605 mμ [2], and then on a photoelectric colorimeter (type FEK M-1) with a yellow filter.

The green suspensions of various kinds of foliage (wheat and rye shoots and sugar-beet and bean leaves) were prepared by grinding a batch of material in the cold with 0.87% potassium dihydrogen phosphate and then centrifuging it for 10 to 15 minutes at 2000 to 2500 r.p.m. The suspension was diluted with buffer because when measured on the photoelectric colorimeter its extinction E was about 2 when measured against water. The suspension thus obtained was stable and its turbidity did not change on prolonged standing, a quality necessary for carrying out the measurement.

In order to carry out the measurements in a more sensitive range of the apparatus (E = 0.7 to 0.3), we introduced into the light beam entering the photoelement a one-centimeter-thick cuvette with a green solution of the same suspension diluted 1.5 times with buffer. Thus the measurement was carried out not against water, but against this suspension.

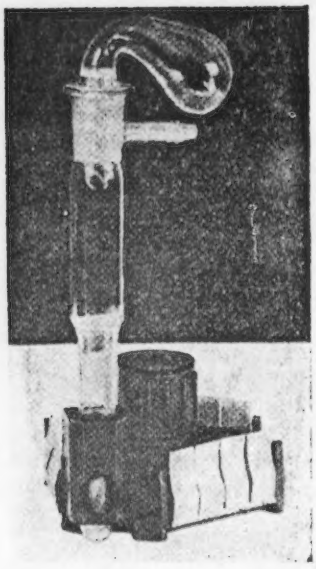

Fig. 2. Arrangement of the support of the Thunberg tubes in the cuvette holder.

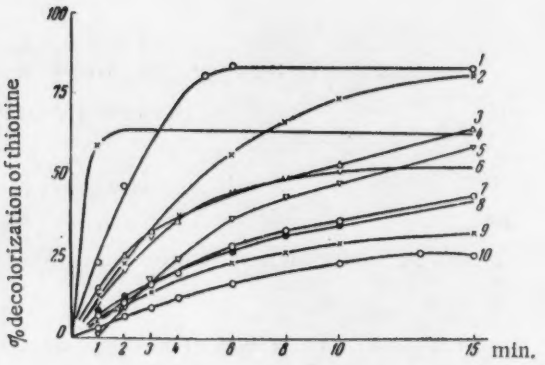

Fig. 3. Dehydrogenase activity of green suspensions of bean leaves. Hydrogen donors: 1) glutamic acid; 2) citric acid; 3) fumaric acid; 4) hexose diphosphate; 5) glucose; 6) histidine; 7) succinic acid; 8) malic acid; 9) glycerophosphate; 10) glycine.

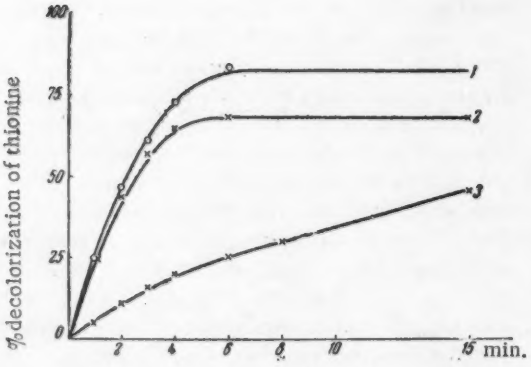

Fig. 4. Dehydrogenase activity of the glutamic acid in a green suspension of rye shoots, of bean leaves, and sugar leaves. 1) Bean leaves; 2) rye shoots; 3) sugar beet leaves.

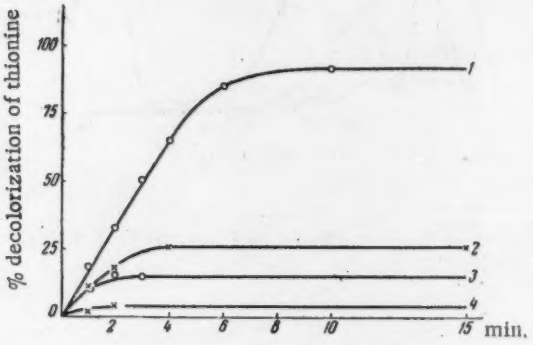

Fig. 5. Dehydrogenase activity of the glutamic acid in a suspension of bean seeds, determined with thionine and methylene blue. 1) With thionine; 2) with methylene blue; 3) boiled suspension with thionine; 4) boiled suspension with methylene blue.

The dehydrogenase activity was determined at 37 to 39° in Thunberg tubes with a lower diameter of 1.5 cm. In order to fix the position of the tubes firmly in the cuvette holder, a special support was mounted, which was placed close to the optical planes of the apparatus (left light beam ; Fig. 2).

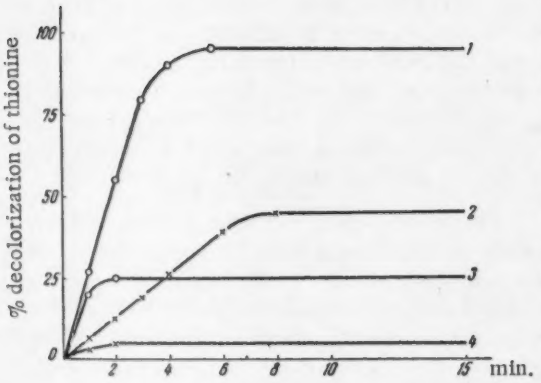

Fig. 6. Dehydrogenase activity of the glutamic acid in a suspension of wheat seeds, determined with thionine and with methylene blue. 1) With thionine; 2) with methylene blue; 3) boiled suspension with thionine; 4) boiled suspension with methylene blue.

The hydrogen donors: glucose, glycerophosphate, hexose diphosphate, glutamic, malic, succinic, citric and fumaric acids, histidine and glycine, were used in the form of 0.1 M solutions neutralized by potassium hydroxide according to phenolphthalein.

For a determination we placed 4 ml of the green suspension and 1 ml of the donor solution in the Thunberg tube and 4 ml of thionine in the head (E = 1.2 as measured through the yellow filter against water). The air was then pumped out for 2 to 3 minutes with an oil pump. The tubes were stabilized at 37 to 39°C for 10 minutes, after which the thionine was added and the extinction measured.

For an initial measurement we used E immediately after pouring in the thionine, and subsequent measurements were made at 1-, 2- and 5-minute intervals for 20 to 30 minutes.

The initial measurement of E on pouring in the thionine was taken as 100%. On complete decolorization of the thionine, the decrease in E amounted to 53% of initial amount. This decrease (53%) was taken as 100% for further calculations.

Control experiments with boiled suspensions in many cases showed a considerable initial speed of the reaction, which, however, very quickly stopped. This phenomenon, in all probability, was caused by the presence of non-enzymatic processes being brought about by the products of cell metabolism, for example, ascorbic acid. Figure 4 shows the curves of the dehydrogenase activity of the glutamic acid of a green suspension of rye shoots, bean leaves, and sugar beet leaves. The dehydrogenases of suspensions of various types of foliage have difference degrees of activity.

In order to compare the results of the determination of dehydrogenase activity according to the thionine and the methylene blue methods, we made a spectrophotometric determination of the dehydrogenase activity of glutamic acid in suspensions of bean seeds and wheat with these dyes (Figs. 5 and 6).

The extinction with methylene blue (with a red filter) was 1.25, that is, less than is usual for the visual method. With thionine the reaction goes further than with methylene blue, which is fully in accord with the value of the oxidation-reduction potential of these compounds (thionine is more easily reduced). In the control experiments with a boiled suspension in the presence of methylene blue, as well as of thionine, in the first minute there is observed a certain decrease in extinction, after which the process stops.

Simultaneously with the spectrophotometric measurement of the activity by methylene blue, we observed this process visually, using time of decolorization. The moment of decolorization evidently coincided with the cessation of change in the extinction. The small change in extinction which we observed in the boiled suspension could not be observed visually.

We expressed the dehydrogenase activity in percent of decolorization of thionine or methylene blue after 5 minutes. After this time the process of decolorization has already stopped; however, the initial speed of the non-enzymatic process appears to be only in the first minute, which does not introduce a significant error into the five-minute determination.

To compare the dehydrogenase activity of various substances we calculated the amount of dye decolorized in μg per mg of protein. The amount of the dye was calculated according to the formula:

$$a = \frac{EnM10^3}{E_M}$$

Dehydrogenase activity of growing substances

Substance	Protein mg/ml suspension	Dry weight, mg/ml suspension	Dehydrogenase activity % decolorization of thionine after 5 min.			Dehydrogenase activity µg. decolorized thionine per mg protein			Dehydrogenase activity % decolorization of methylene glue after 5 min.	Dehydrogenase activity in µg. decolorized methylene blue per mg protein.
			glutamic	citric	malic	glutamic	citric	malic	Glutamic	
Bean leaves	0.997	11.5	80	51	25	1.23	0.78	0.384		
Wheat shoots	0.9362	9.6	45	16	22	1.47	0.52	0.68		
Rye shoots	0.4787	9.9	67	40	46	2.14	1.28	2.94		
Sugar beet leaves	0.332	3.8	23	–	–	1.06	–	–		
Bean seeds	2.409	26.0	74	87	77	0.94	1.10	0.97	22	0.30
Wheat seeds	1.0762	12.5	76	–	77	2.16	–	2.18	34	1.04

in which a is the quantity of dye, in µg, dissolved in n ml, E is the extinction of thionine at 605 mµ = 1 (1.20 on the photoelectric colorimeter) and methylene blue at 665 mµ = 0.765 (1.25 on the photoelectric colorimeter, M is the molecular weight (thionine, 333; methylene blue, 319), E_M is the molecular extinction (for thionine, 1.09×10^5 at 605 mµ; for methylene blue, 0.74×10^5 at 655 mµ). The data are given in the table.

The positive aspects of the spectrophotometric method shown by the experiments described above, are: it makes possible the determination of the dehydrogenase activity directly in the green suspensions with a minimum lose of the material in solution (enough suspension is obtained from 1 g of bean leaves for seven determinations); the method is shown to be objective; and it allows measurement of the kinetics of decolorization of thionine. Stability of the suspension is indispensable for carrying out the measurement.

A defect of the method is the fact that it does not enable us to determine the full activity of the dehydrogenase of all the tissue, since the dehydrogenase is determined in the centrifugate without taking into account the activity of the precipitate.

We conclude with an expression of warm appreciation to N. M. Siskyan and A. A. Krasnovsky for their direction of the work and discussion of the results.

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SOME PROPERTIES OF LITMOCIDIN

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In 1945 Gauze [1] isolated an actinomycete from the south of Russia, Proactinomyces cyaneus antibioticus, which forms an antibiotic substance, named litmocidin in view of its property, like litmus, of changing color, depending on the reaction medium. Brazhnikova [2] succeeded in isolating the pure active principle of litmocidin and established that it is a pigment and has many reactions in common with anthocyanidins. The present work is confined to the general chemical characterization of litmocidin.

We established that the interval of color transition of litmocidin, determined by the buffer mixtures according to Sørensen, lies within the limits pH 6.4 to 7.7 (see table).

Color of litmocidin as a function pH

pH	6.2	6.4–6.9	7.00	7.1–7.7	8.0
Color of litmocidin	red	reddish-purple	purple	bluish purple	blue

Litmocidin is not a glycoside, since no carbohydrates were found among the products of its acid hydrolysis.

All known anthocyanidins, which are oxonium bases, form red-colored salts with strong mineral acids. Since we obtained litmocidin in the presence of hydrochloric acid, we assume that we are dealing with its hydrochloride salt. However, qualitative analysis did not show the presence of the chlorine ion in carefully washed preparations of litmocidin. Combustion showed that litmocidin contains only traces of chlorine, but also sulfur, phosphorus and nitrogen. Consequently, litmocidin does not represent an oxonium salt. We did not succeed in obtaining litmocidin in crystalline form. The picrate of litmocidin was obtained from its derivatives; it was red in color and had a melting point of 100 to 102°C. Dilute aqueous solutions of litmocidin are turned to violet by aqueous solutions of ferric chloride, which attests to the presence of a phenol.

As Brazhnikova showed [2], nascent hydrogen decolorizes acidic aqueous solutions of litmocidin; the color returns if the solution is left standing in air. On standing for a very long period in air, an aqueous solution of litmocidin loses its color, which does not reappear. Decolorization with hydrogen peroxide proceeds very slowly and without regeneration of the color. Alcoholic solutions of litmocidin are very stable.

Elementary analysis of litmocidin gave the following results:

C– 60.05%; 59.94%; 59.92%		C– 59.6%
H– 5.32%; 5.38% 5.40%	Average	H– 5.36%
		O– 33.51%
	Cl, S, P, N,	– 1.17%
		100.00%

The molecular weight of litmocidin was determined by cryoscopy in various solutions according to Beckmann's procedure.

During cryoscopy in naphthalene [3] we observed that the depression varied in the course of a single experiment. The calculated molecular weight was therefore correspondingly increased, and expressed in the following series of figures:

1) For a weight of 0.112 g : 423, 434, 465, 505
2) For a weight of 0.159 g : 418, 460, 503
3) For a weight of 0.25 g : 454, 488, 509, 532

Thus, the minimum molecular weight of litmocidin found in naphthalene was 418. Without attempting to interpret this phenomenon, we determined the molecular weight of litmocidin in the same way, but using phenol, and found it to be 398. Grafe [4] and Wheldale [5], who determined the molecular weight of anthocyanidins by using cryoscopy in phenol, obtained results somewhat too low by this method. Assuming that in the determination of the molecular weight of litmocidin in phenol we obtained its lowest value, it is possible to consider that the actual molecular weight of litmocidin lies within the limits 398 to 418. The empirical formula of litmocidin, calculated from the data of the elementary analysis and of the molecular weight, is $C_{20}H_{22}O_9$.

Spectroscopic analysis showed that litmocidin has three absorption maxima, in the yellow, green and blue portions of the spectra, with the following wavelengths: 1) 570 to 560 mμ; 2) 530 to 510 mμ; 3) 480 to 460 mμ.

The strong color of its solutions interfered with determination of the optical activity of litmocidin.

The number of atoms of active hydrogen was determined in the laboratory of Prof. A. P. Terentyev by the method of Terent and Shor [6], using methylzinc iodide. Active hydrogen was found to be 2.27%, which corresponds to 9 atoms of active hydrogen for one molecule of litmocidin. The considerable content of atoms of active hydrogen may be explained by the presence in the litmocidin molecule of hydroxyl and possibly carboxyl groups. The assumption regarding the presence of carboxyl groups was confirmed both by the acid reaction of litmocidin solutions and by a number of properties inherent in carbonic acids. Among the latter properties is the ability of litmocidin to decompose carbonates and to dissolve in organic solvent with an acid reaction and in aqueous solvent with an alkaline reaction, forming alkaline salts which are readily soluble in water.

To determine the number of carboxyl groups, alcoholic or alcohol-and-water solutions of litmocidin were titrated by weak alkali; the end point of the titration was determined by a change in color of the solution from red to blue. Only in the titration of very dilute solutions of litmocidin (containing 4 to 15 mg. by weight) with 0.005 N KOH could the end point be established with sufficient precision. On titrating to the violet coloration (pH 7.0), 10 to 14% of carboxyl groups was found, which , at a molecular weight of 398 to 418, corresponds to approximately one carboxyl group per molecule. On titrating to the blue color (pH 8.0), 20 to 23% carboxyl groups was found, which corresponds to two carboxyl groups. Titration by more concentrated solutions was made difficult by their strong coloration.

The use of conductometric titration, which is usual for colored solutions, did not give satisfactory results, since a whole series of acid organic compounds containing organic hydroxyl groups behave like the carboxylic acids; the question of the presence of carboxyl groups in litmocidin requires more careful proofs.

To obtain an idea of the structure of litmocidin, we attempted to recover products of its decomposition by fusing with strong alkali. Willstaetter [7], and after him, Karrer and Widmer [8], established the structure of many anthocyanidins by fusing them with strong alkali, and showed that characteristic products of their alkaline hydrolysis are phloroglucinol and aromatic hydroxy-acids. The latter are best recovered by Karrer's method.

We carried out four alkaline hydrolyses of litmocidin: three by Willstaetter's method, fusion of litmocidin with strong alkali at 220 to 250°C and one by Karrer's method. We carried out the hydrolysis by the latter method in a current of hydrogen, replacing the strong alkali by a 15% solution of it, and lowering the temperature. Each hydrolysis yielded two fractions, an acid and an alkaline fraction, in extremely small quantities.

Both fractions appeated to be sticky, oily residues, poorly soluble in water but quite soluble in alcohol. The most sensitive tests did not reveal even a trace of phloroglucinol in the basic fraction. The acid component of the hydrolyzate gave a reddish-brown color with ferric chloride, which indicated the presence in it of a substance containing phenolic hydroxyls. We could not identify these fractions because of the impossibility of obtaining them in crystalline form.

From all the preceding it appears that, judging by its antibacterial properties, how it is produced, its molecular weight, the number of atoms of active hydrogen, and the absence of phloroglucinol in the products of its alkaline decomposition, litmocidin is different from all other anthocyanidins known at the present time. The litmocidin molecule is considerably bigger than that of the usual anthocyanidins. The fact that the absorption spectrum of litmocidin is close to that of most anthocyanidins testifies to the presence in them of common structural characteristics.

SUMMARY

The antibiotic litmocidin has a molecular weight lying within the range 398 to 418; the empirical formula, calculated from elementary analysis data and from the demonstrated molecular weight, is $C_{20}H_{22}O_9$. Litmocidin has three absorption maxima in the visible portion of the spectrum with wavelengths of 570 to 560, 530 to 510, and 480 to 460 mμ, and contains 9 active hydrogen atoms. Litmocidin is not a glycoside, since its acid hydrolysates do not contain carbohydrates. Litmocidin does not form salts with mineral acids. Phloroglucinol was not observed among the products of its alkaline hydrolysis.

According to all the properties enumerated above, litmocidin is different from all anthocyanidins known at present. The similarity of the absorption spectra of litmocidin and of anthocyanidins suggests the presence in them of common structural characteristics.

Deep gratitude is expressed to Prof. A. N. Belozersky for his supervision of the present work.

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STUDY OF THE ENZYMATIC CONVERSION OF STARCH IN POTATO TUBERS AT AT VARIOUS PERIODS OF VEGETATION

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Previously, in experiments with extracts, we showed that as potato tubers ripen there is generated within them enzymatic activity of such a nature that they are able to accumulate starch [1]. At the same time that there is a noticeable diminution of the splitting action of extracts, the synthesizing action either remains at the same level or increases [2]. During this process an increase in the starch content and a noticeable decrease in the content of reducing substances takes place in the tubers.

It was of interest to study the action of the individual enzyme systems taking part in these processes.

In the present work are shown data from studies of the hydrolytic and phosphorolytic action of potato tuber extracts at various periods of vegetation, as well as of the synthesizing and phosphoglucomutase activity, since, as our investigations showed [3], the synthesizing action of phosphorylase and the action of phosphoglucomutase are closely interconnected. The starch content and the content of reducing substances in the extracts were determined at the same time as the enzymatic activity.

METHOD

From potato tubers, extracts [4] were prepared which were studied at two periods: I) In July, at the time when the potatoes were flowering and when they had small tubers; and II) after harvesting the crop. The phosphorolytic and hydrolytic actions of the extracts were determined after dialysis for 16 to 18 hours; for the study of phosphorolysis, phosphate was added to the dialyzate, while in studying hydrolysis, chloride was added to the dialyzate. In the first case the loss in inorganic phosphate was determined; in the second case, the increase in the products of hydrolysis, i.e. the reducing substances, was determined.

At the same time a quantitative determination was made in non-dialyzed extracts, immediately after preparation, of phosphate esters of reducing substances and of reducing substances not containing phosphorus (free sugars). The first group of substances was separated from the second group by precipitation with alcohol in the presence of barium. The alcoholic extract was evaporated to dryness on the water bath, the residue dissolved in water, and in the aqueous solution we determined the quantity of reducing substances not containing phosphorus [5].

The determination of the synthesizing and of the phosphoglucomutase activity (the reaction converting glucose-1-phosphate into glucose-6-phosphate) was carried out according to methods previously described [2, 3].

The phosphorylase fraction was isolated from potato extract by precipitation with ammonium sulfate (28 g per 100 ml of extract at 30°). The precipitate obtained was dissolved in 0.1 N citrate buffer at pH 6.5 and the solution obtained was dialyzed against water for 24 hours and then subjected to investigation.

The starch content of the tubers was studied enzymatically. The investigations were carried out on three varieties of potatoes: "Lorkh," "Preskulsky," and "Oktyabrenok." Because identical results were obtained with all varieties, experimental data are shown for the decomposition in potato tubers of the "Lorkh" variety, while investigations of the synthesizing and phosphoglucomutase activity used the "Oktyabrenok" variety.

Study of the Decomposition of Starch

Experimental data for the phosphorolytic and hydrolytic action of extracts, and also the starch content of extracts, is shown in Table 1 and in the figure.

TABLE 1

Study of the Phosphorolytic Action of Extracts of Potato Tubers. Incubation mixture: 2 ml extract + 0.5 ml 2% starch solution + + 0.3 ml phosphate buffer + 0.3 ml 0.1 N NaF. Loss in inorganic phosphate is expressed in μg per ml of extract.

Period I			Period II		
Incubation time in hours					
0	0.5	2	0	0.5	2
0	200	250	0	60	150
0	190	240	0	135	220
0	150	249	0	160	160
0	160	240	0	85	180
0	120	220	0	115	200
0	100	250	0	90	235

From the preceding data it is seen that the phosphorolytic action of tuber extracts decreased in proportion to the ripening of the tubers. Thus, the loss of inorganic phosphate in the first period of vegetation was more pronounced than in the second; the loss of phosphorus in the first period was greater after both 30 minutes and 2 hours of incubation. The greatest difference in phosphorolytic activity was observed after 30 minutes, which shows a decrease in the velocity of phosphorolysis in ripe tubers. However, even after a longer incubation period, the phosphorolytic splitting in ripe tubers did not attain the level achieved by the same process in tubers in the first period. These data show that the phosphorolytic splitting process in ripe tubers was slowed down as a whole.

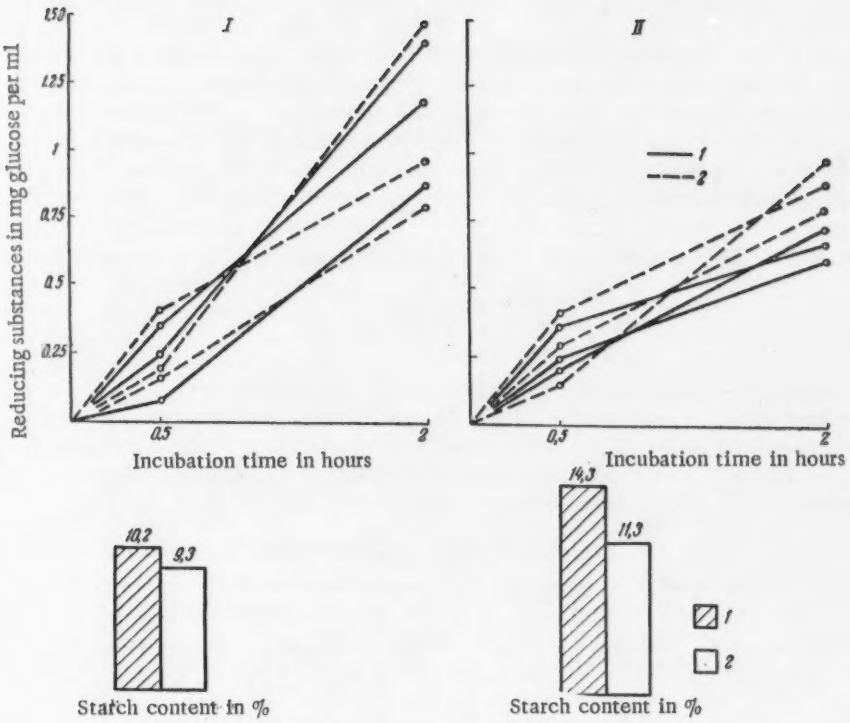
On studying the hydrolytic activity of extracts we observed the same pattern: decrease of hydrolytic activity on ripening of the tuber (see figure). The curves of the accumulation of reducing substances are lower than they are in the first period of investigation. Decrease in hydrolytic action was more strongly pronounced in the first experiment than in the second, even though the hydrolysis took place at the same rate in the first as in the second. A more pronounced decrease in hydrolytic activity was observed specifically in those tubers which contained a greater quantity of starch; as the tubers ripened their starch content in the first experiment was considerably higher than in the second. The differences in starch content of tubers of the same variety—"Lorkh"—was apparently caused by the use in the soil of different fertilizers: in the first experiment we used N_{90}, K_{120} (kainit), and in the second, N_{25}, P_{90}, K_{120} (potassium-magnesium).

Thus, the experiments carried out showed that when tubers ripen, the hydrolytic activity of extracts of these tubers decreases. This decrease is greater in those tubers in which a greater accumulation of starch is taking place.

During the study of reducing substances in the same tubers extracts we observed a condition which we had already described in part [1]: i.e., as the tubers ripened, a decrease took place within them of the total quantity of reducing substances. In the present study, we used fractionation to separate the two groups of reducing substances: 1) those not containing phosphorus (substances extracted by alcohol); and 2) reducing phosphate compounds (precipitated substances). From the data shown in Table 2 it is seen that the main body of the reducing substances in tubers is found in the first group (97 to 83%).

The quantity of phosphorus-containing reducing compounds in extracts is very small, amounting only to about 3 to 8% of the total quantity of reducing substances. The data obtained show also that the quantity of reducing substances in ripening tubers (period II) decreases abruptly, in which process their decrease takes place entirely at the expense of substances not containing phosphorus. Phosphorus-containing reducing compounds remain unchanged in this process, and the amount of their reduction remains approximately the same in both the first and second experimental periods.

Thus, during the ripening of tubers and the accumulation within them of starch, an abrupt decrease is observed in the amount of non-phosphorus-containing reducing substances constituting the basic group of all reducing substances in tubers, while phosphorus-containing reducing substances remain unchanged.



Study of hydolytic activity and of starch content in potato tubers: I) Period I; II) Period II. 1) Experiment 1; 2) Experiment 2. See text for explanation of designations.

TABLE 2

Study of Reducing Substances in Potato Tubers

Quantity of reducing substances in mg of glucose per ml

Period I			Period II		
Total quantity	Not containing phosphorus	Phosphorus compounds	Total quantity	Not containing phosphorus	Phosphorus compounds
3.07	2.76	0.107	1.72	1.62	0.140
3.9	3,8	0,142	2.25	1.89	0.130
4.27	4.15	0.190	2.25	1.88	0.126
2.17	2.0	0.101	1.5	1.5	0.150
4.5	4.21	0.123	1.87	1.66	0.127
4.4	4.5	0.09	2.15	1.86	0.142

Study of the Synthesizing Action of Phosphorylase and Phosphoglucomutase Activity In Extracts

As our previous investigations showed, the synthesizing action of phosphorylase is intimately related to the activity of the phosphoglucomutase which catalyzes the conversion of glucose-1-phosphate to glucose-6-phosphate. Both enzymes, acting on the same substrate, show different actions, and a decrease in the activity of one enzyme brings about an increase in the activity of the other. In the ripening of tubers, the activity of phosphoglucomutase is decreased, while the synthesizing action of phosphorylase is increased. During storage of the tubers, the phosphoglucomutase activity decreases abruptly or is completely suppressed, while the synthesizing action increases strongly [4]. The activity of phosphoglucomutase in storage, however, may be fully restored by the addition to its extracts of the activators Na_2SO_3 and $MgSO_4$, whereupon the synthesizing action declines sharply.

TABLE 3

Study of the Conversion of Glucose-1-phosphate in Potato Tuber Extracts
Incubation mixture A: 1 ml extract + 21 mg glucose-1-phosphate + 5 mg glycogen + H_2O to make 3 ml; mixture B: same as A but with the addition of 7.5 mg Na_2SO_3 + 3.75 mg $MgSO_4$ + 1 mg veronal buffer, pH 7.5 to 7.7; temperature, 38°; a) conversion of glucose-1-phosphate into polysaccharide in %; b) conversion of glucose-1-phosphate into glucose-6-phosphate in %.

A. Experiments Without Phosphoglucomutase Activator

a) Period I				b) Period II			
a		b		a		b	
30 min.	2 hrs.	30 min.	2 hrs.	30 min.	2 hrs.	30 min.	2 hrs.
36	36	20	60	22	60	6	23
20	26	13	65	29	51	12	35
34	40	25	60	24	32	26	54

B. Experiments with phosphoglucomutase activator

10	22	24	70	20	37	14	57
10	8	43	87	8	33	16	60
22	22	23	78	12	34	12	61

TABLE 4

Study of the Active Fraction of Phosphorylase
A) Synthesizing action of phosphorylase in %; B) phosphoglucomutase activity in %.
Incubation mixture: 1) 1 ml phosphorylase + 21 mg glucose-1-phosphate + 5 mg glycogen + 1 ml citrate buffer + 1 ml 0.04 N NaF; 2) the same + phosphoglucomutase activator.

No.	A			B		
	0	30 min.	120 min.	0	30 min.	120 min.
1	0	66	82	0	0	0
2	0	56	74	0	0	7

In the present studies it is shown that in the vegative stage of potatoes, the ratio of the activities of phosphoglucomutase and phosphorylase (being regulated, probably, by a number of factors) depends largely on the condition of the phosphoglucomutase activators. Experimental studies of the phosphorylase and phosphoglucomutase action of extracts were carried out with and without phosphoglucomutase activators. The results of these studies, shown in Table 3, indicate that the addtion of activators to the tuber extract in both the first and second periods of the investigation leads to a decrease in the synthesizing action of phosphorylase and to an increase in the phosphoglucomutase action.

Thus, in the first period, the activity of phosphorylase without activators amounted to 20 to 36% (after 30 minutes of incubation) and 26 to 40% (after 2 hours), while with the addition of activators it decreased to 10 to 22% (after 30 mins) and 8 to 22% (after 2 hours), that is, a decrease of about 2 times. Such a decrease in the synthesizing action under the influence of activators was observed in the second experimental period. In contrast to this, the phosphoglucomutase activity increased: in the first period from 13 to 25% it grew to 23 to 43% (after 30 minutes of incubation) and from 60 to 65% to 70 to 87% (after two hours). This was approximately the nature of the increase in phosphoglucomutase activity which we observed in the second period.

Thus these investigations showed that the synthesizing action of phosphorylase is closely dependent on the activiating system for phosphoglucomutase. The question arose: do the phosphoglucomutase activators exert a directly inhibiting action on the phosphorylase, or is this inhibiting action a result of the increase in phosphoglucomutase action as a consequence of which a definite quantity of glucose-1-phosphate is, so to speak, removed from the sphere of action of phosphorylase? To answer this question we isolated the phosphorylase fraction and studied the direct action on its activity of the activators.

The data from this investigation, which are shown in Table 4, indicate first that the isolated fraction contained active phosphorylase and only traces of phosphoglucomutase. Also, from the data shown it appears that the activators show almost no inhibiting action on the phosphorylase, or else only to an extremely slight degree. Thus, these direct experiments show that the phosphoglucomutase activators exert an inhibiting action on the synthesizing activity of phosphorylase not directly, but through phosphoglucomutase. The increase in the synthesizing action of phosphorylase during ripening of potato tubers is related to the suppression of action of the activators of phosphoglucomutase during this period of vegetation.

DISCUSSION OF RESULTS

Our investigations showed that when potato tubers ripen, a decrease is observed in the quantity of reducing substances, and a completely clear suppression of the activity of the phosphorolytic and hydrolytic enzyme systems is also seen. These data testify to the importance of such systems in the storage of starch in the period of ripening of the tuber.

However, we assume that in the enzymatic reaction starch $\rightleftharpoons$ reducing substance, a significant role is played not only by the enzymes acting directly on starch, but also by the enzymes causing conversion of the free sugars. The significance of the free sugars in the processes related to the synthesis of starch is confirmed by the large content of these substances in tubers (alcoholic extracts contain a preponderance of free sugar [1]) and by a decrease in the quantity of them during the accumulation of starch in the tubers.

A study of the synthesis of starch under the action of phosphorylase showed that an important part is played by phosphoglucomutase, which was first observed in potato tubers by Sisakyan [6]. Out studies showed that the increase in the synthesizing activity of phosphorylase when tubers ripen is related to the decrease in phosphoglucomutase activity which, in turn, depends on its activators. Thus, the phosphoglucomutase activators are an important factor in the regulation of the synthesizing action of the phosphorylase of potato tubers.

SUMMARY

When potato tubers ripen, the phosphorolytic and hydrolytic action of extracts of these tubers declines. One of the important factors in increasing the synthesizing action and decreasing the phosphoglucomutase activity of tuber extracts is the decrease in action of the phosphoglucomutase activators.

The basic group of reducing substances in tubers consists of compounds which do not contain phosphorus. In proportion to the ripening of the tubers, when starch is accumulating within them, there is decrease in the content of non-phosphorus-containing reducing substances, but the quantity of phosphorus-containing reducing compounds changes very little or not at all in the process.

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THE CHANGE IN THE SYNTHESIS AND TRANSAMINATION OF AMINO ACIDS IN RAT LIVER IN PROTEIN DEFICIENCY

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Previously published data [1] show that transamination (glutamic acid-alanine transaminase) within liver slices during protein deficiency in the rat is considerably disrupted, while this deficiency has no effect on the synthesis of amino acids from keto acids and ammonium salts.

In the works of Awapara [2] and of Caldwell and McHenry [3] data are also shown relating to the disturbance of transamination during protein deficiency; here it was found to be principally the transamination between alanine and α-ketoglutaric acid which was disturbed. The enzyme system catalyzing the transfer of amino groups between aspartic acid and α-ketoglutaric acid, according to these authors' data, is almost undisturbed. However, in these authors' experiments the animals were maintained on a diet with more protein (8%) than in our experiments (3%) or on a diet completely without protein, and for this reason it is difficult to compare their data with ours. As far as concerns the effect of protein deficiency on enzyme systems catalyzing the synthesis of amino acids from keto acids, no new data on this question have been revealed in the literature in recent years. However, while studying the synthesis of amino acids chromatographically, we were able in certain cases to confirm the weakening of this synthesis in animals on a diet with scanty protein.

These data stimulated us to carry out systematic new studies on the effect of protein deficiency on the processes of transamination and synthesis of amino acids, using both the methods which we used before and also paper chromatography.

EXPERIMENTAL METHODS

The experiments were made on white rats on a diet containing 3% protein. The composition of the diet was the same as in the previous study [1]. After the development in the animals of acute phenomena of protein deficiency (loss of weight of 25 to 30%, hypoproteinemia, decrease in the urea content of the urine, etc.) the animals were killed and, to determine the rate of synthesis of amino acids, the liver was prepared in slices which were incubated with various keto acids in bicarbonate buffer (pH 7.4) in an atmosphere of 95% O_2 + 5% CO_2 at a temperature of 37°. Each sample contained 250 mg of slices and neutralized substrates in the following concentrations: pyruvic acid, 0.1 M; α-ketoglutaric acid and oxaloacetic acid, 0.05 M; ammonium carbonate, 0.02 M. At the end of incubation, the proteins were precipitated with 20% trichloroacetic acid, the filtrate freed from the latter by ether extraction, and the amino acids formed determined by paper chromatography using water-saturated phenol as the mobile phase or quantitatively by Van Slyke's method.

For experiments in the determination of the rate of transamination between α-ketoglutaric acid and aspartic acid and between α-ketoglutaric acid and alanine, we used a 20% liver homogenate and these substrates at a concentration of 0.1 M for samples. We carried out parallel control procedures on the livers of rats kept on a normal diet along with all these experiments.

RESULTS

Figures 1 to 3 show chromatograms of the results of our studies of the synthesis of amino acids using as substrates pyruvic, α-ketoglutaric, and oxaloacetic acids.

From Fig. 1 it is seen that in the synthesis of amino acids from pyruvic acid, the alanine spots in the experiments with slices of liver from rats on a low-protein diet (Sample 5) are weaker than the corresponding spots in experiments with liver slices from normal rats (Sample 1). A definite decrease in the rate of formation of glutamic acid from α-ketoglutaric acid is also seen in the chromatogram in Fig. 2 (Samples 5 and 1). In the synthesis of aspartic acid from oxaloacetic acid, a clear answer could not in this case be obtained from the chromatogram. However, from Tables 1, 2 and 3, containing data relative to the increase in amino nitrogen, it is seen that with a protein-deficient diet there is a considerable decrease in the rate of formation of amino acids using as substrates for amination all three of the keto-acids studied.

TABLE 1

Synthesis of Amino Acids from Pyruvic Acid and $(NH_4)_2CO_3$ by Rat Liver Slices

Normal rats		Rats on protein-deficient diet	
Serum protein, %	Increase in amino N, μ moles/g of tissue	Serum protein, %	Increase in amino N, μmoles/g of tissue
8.0	308	5.2	60
7.6	221	5.5	47
–	–	5.4	110
7.0	160	5.4	50
6.3	220	5.2	46
6.6	142	–	28
6.5	176	4.1	47
–	194	4.5	87
Average:	203	Average:	59

TABLE 2

Synthesis of Amino Acids from α-Ketoglutaric Acid and $(NH_4)_2CO_3$ by Rat Liver Slices

	Normal rats	Rats on protein-deficient diet		
Serum protein, %	Increase in amino N, μmoles/g of tissue	Weight loss, %	Serium protein, %	Increase in amino N, μ moles/g tissue
6.5	79	30	5.1	38
6.5	98	17	5.9	33
6.7	113	18	4.5	36
7.0	93	–	–	–
–	138	22	5.4	77
–	134	19	5.6	65
6.1	95	–	4.8	59
7.5	110	–	5.2	51
Average:	108		Average:	51

Thus, in distinction to the data shown in our first communication, we found that when the animals were maintained for a long time on a protein-deficient diet, there are disturbances also of the enzyme system catalyzing the synthesis of amino acids from the keto acids studied and ammonia. In this process the greatest disturbance

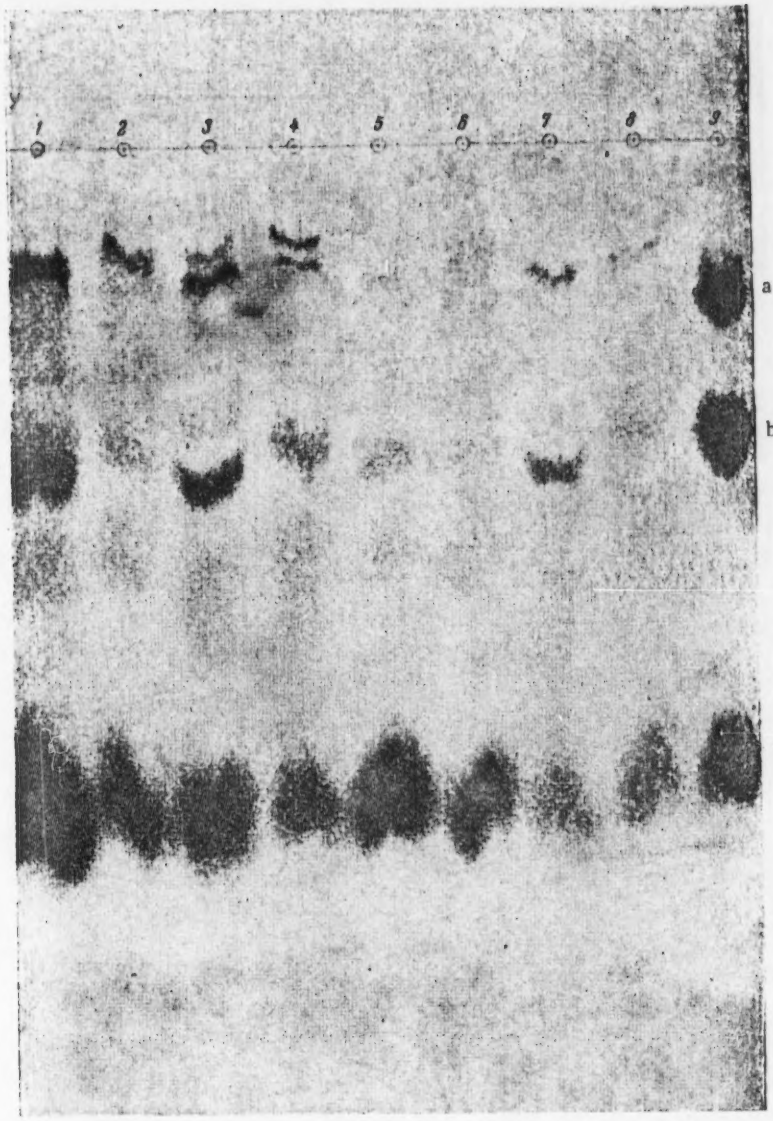

Fig. 1. Formation of amino acids in liver sections of a normal rat (1,2,3 and 4) and of rat on a low protein diet (5, 6, 7 and 8); the sections were incubated for a period of two hours. Substrate: in tests 1, 5, pyruvic acid and $(NH_4)_2CO_3$; tests 2 and 6, pyruvic acid; test 3 and 7, $(NH_4)_2CO_3$; tests 4 and 8, Ringer-bicarbonate; test 9, indicator: a) aspartic acid b) glutamic acid, and c) alanine, 6 μg. The filtrates deposited upon the chromatogram corresponded to 1.5 mg of the original tissue.

was that noted in the amination of pyruvic and oxaloacetic acids (the rate decreased by approximately 3 times), while the amination of α-ketoglutaric acid was less disturbed (decreased by 2 times).

The data shown in Figs. 1 to 3 also make it possible to consider the effect of protein deficiency on the transamination of amino acids as well. Actually, in samples associated with experiments with normal rats, in addition to spots of each basic amino acid being formed, there are also seen distinct spots of certain amino acids the formation of which, apparently, is a result of transamination among the amino acids being formed from the added keto acids, and by the keto acids previously formed in the liver.

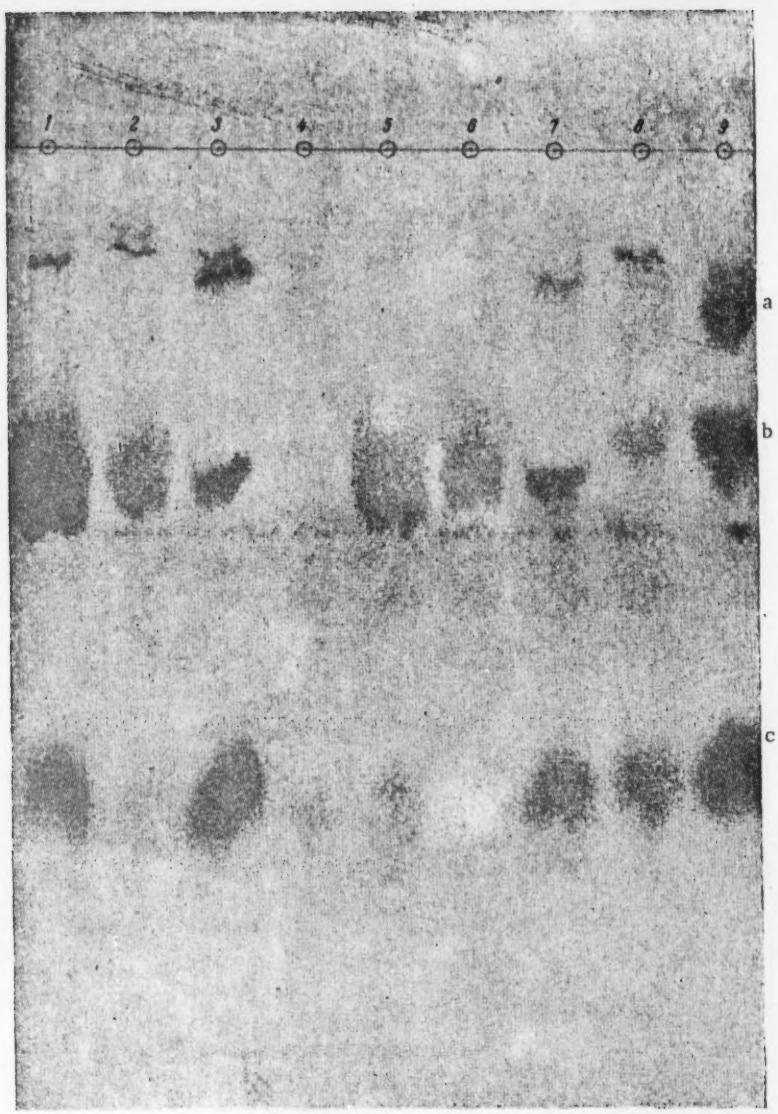

Fig. 2. Formation of amino acids in liver sections of a normal rat (1,2,3, and 4) and of a rat on a low protein diet (5,6,7, and 8); the sections were incubated for a period of two hours. Substrate: in tests 1 and 5, ketoglutaric acid and $(NH_4)_2CO_3$; tests 2 and 6, ketoglutaric acid; tests 3 and 7, $(NH_4)_2CO_3$; tests 4 and 8, Ringer-bicarbonate; test 9, indicator: a) aspartic acid, b) glutamic acid, and c) alanine.

Thus, for example, in Fig. 1 (Sample 1) along with a sharply outlined spot of alanine formed from pyruvic acid are also seen distinct spots of glutamic acid and aspartic acid. In the same way in Figs. 2 and 3 in the samples taken from normal animals, in addition to the spots of glutamic and aspartic acids, are seen distinct spots of alanine; however, in samples with liver slices from rats on a protein-deficient diet these supplementary spots either appear more faintly or are completely absent. The absence of such spots in samples associated with the experimental animals again confirms the disturbance (which we established in the preceding investigations) in the transamination of amino acids in protein deficiency. This was confirmed in studies of such systems of transamination and in direct experiments with the livers of rats on a protein-deficient diet. The rate of transamination be-

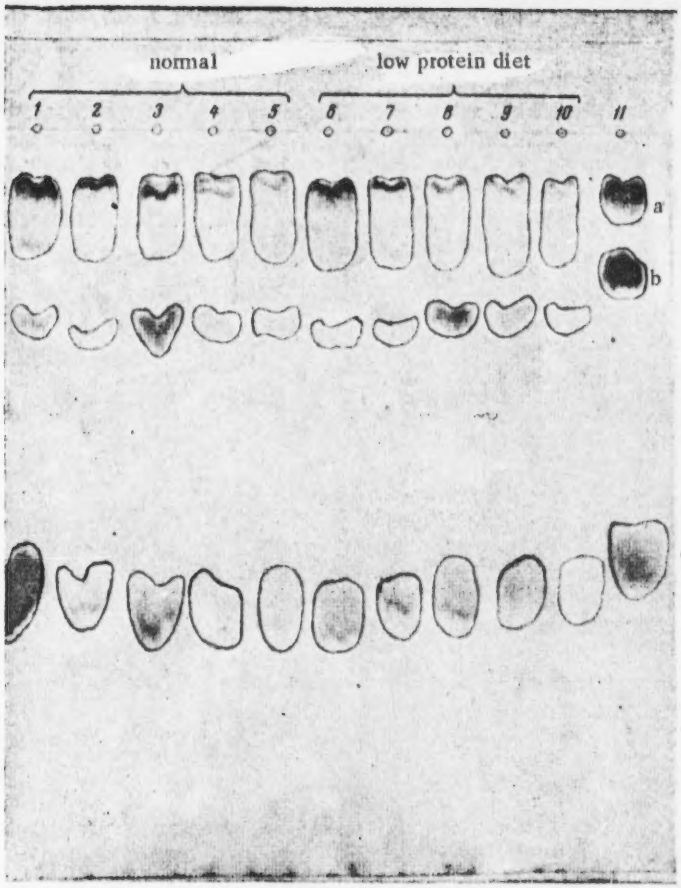

Fig. 3. Formation of amino acids in liver sections of a normal rat (1,2,3,4 and 5) and of a rat on a low protein diet (6,7,8,9, and 10); the sections were incubated for a period of three hours. Substrate: In tests 1 and 6, oxaloacetic acid and $(NH_4)_2CO_3$; tests 2 and 7, oxaloacetic acid; tests 3 and 8, $(NH_4)_2CO_3$; tests 4 and 9, Ringer-bicarbonate; test 5 and 10, oxaloacetic acid and $(NH_4)_2CO_3$, fixed without incubation; test 11, indicators.

TABLE 3

Synthesis of Amino Acids from Oxaloacetic Acid and $(NH_4)_2CO_3$ by Rat Liver Slices

Normal rats		Rats on protein-deficient diet		
Serum protein, %	Increase in amino N, μmoles/g of tissue	Loss of weight, %	Serium protein, %	Increase in amino N, μmoles/g tissue
–	117			–
–	157			–
–	185			–
–	84	21.6	–	24
–	85	20.3	4.8	20
6.8	94	18.0	4.8	36
6.5	166	27.0	5.2	62
7.1	148	24.0	5.9	77
7.6	87	–	5.3	21
Average:	125		Average:	40

TABLE 4

Activity of Glutamic Acid-alanine* and Glutamic-aspartic Acid** Transaminase
Activity is expressed in % of the amino acid on which transamination has been carried out (of the original quantity)

Experiment No.	Normal rats				Rats on protein-deficient diet		
	Serum protein, %	Activity		Loss in weight, %	Serum protein, %	Activity	
		Glutamic acid-alanine system	Glutamic-aspartic acid system			Glutamic acid-alanine system	Glutamic-aspartic acid system
1	–	45.7	30.3	23	5.0	38.0	26.8
2	6.5	41.5	30.3	30	4.2	30.6	21.8
3	6.3	–	28.3	30	3.7	32.1	23.1
4	6.4	40.7	28.1	16	2.9	24.6	19.0
5	6.8	47.5	35.3	23	4.8	39.5	27.2
6	–	43.2	29.4	29	3.6	29.1	22.1
7	–	41.5	30.0	29	3.6	22.8	22.1
Average:		44.4	30.2	–	–	30.9	23.1

* The transamination reaction lasted 1 hour.
** The transamination reaction lasted 10 minutes.

tween aspartic and α-ketoglutaric acids and between alanine and α-ketoglutaric acid was determined quantitatively by the formation of oxaloacetic and pyruvic acids, respectively.

The oxaloacetic acid was determined by the quantity of CO_2 formed after its decomposition by aluminum citrate [4]. Pyruvic acid was determined colorimetrically by its reaction with salicylaldehyde.

From the results of the experiments shown in Table 4 it is seen that the rate of transamination between alanine and α-ketoglutaric acid in liver slices from rats on protein-deficient diets is, in certain cases, 50% lower than the transamination rate in normal rats. The average decrease in the rate of transamination in protein deficiency is 30%. The disturbance of the rate of transamination between aspartic and α-ketoglutaric acids is less pronounced; there is an average decrease of 25%. It must be noted that there is a known correlation between the degree of disturbance of enzyme transamination systems and the decrease in blood protein content. Thus, for example, in Experiments 4 and 7, the lowest protein content was noted in the serum of experimental rats, and in them also was observed the greatest decrease in rate of both transamination systems. And, on the other hand, in those cases in which, regardless of the prolonged maintenance of the animals on the diet, the blood protein remained at a high level (Experiments 1 and 5), the transaminase activity decreased only very insignificantly.

The data which we obtained show that if the animals are kept long enough (2 to 3 months) on a diet of 3% protein then not only is the system of transamination between alanine and α-ketoglutaric acid disturbed, but also that between aspartic and α-ketoglutaric acids. Similar results are seen in the recent work of Srinivasan and Patwardhan [5]. With regard to the rapidity of appearance of these disturbances, we were able in a number of experiments to confirm the data of Awapara, who showed that the disturbance of the glutamic acid-alanine transaminase system begins even a few (4 to 7) days after placing the animals on the low-protein diet, but the disturbance of the glutamic-aspartic acid transaminase system begins, as a rule, considerably later.

SUMMARY

In rats on a diet deficient in protein, the synthesis in liver slices of alanine, glutamic and aspartic acids from the corresponding keto acids is disturbed, while the rate of amination of pyruvic and oxaloacetic acids is decreased to a greater degree than that of α-ketoglutaric acid.

The rate of transamination between alanine and α-ketoglutaric acid in these animals is decreased by an average of 30%, while the rate of transamination between aspartic and α-ketoglutaric acids is decreased by 25%.

Inhibition of the first of these systems begins 4 to 5 days after transferring the animals to a protein-deficient diet, while inhibition of the second begins, as a rule, considerably later.

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THE PROTEOLYTIC ACTION OF RENIN PREPARATIONS

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Regardless of the great progress which has been attained in recent years in purifying renin [1] and its substrate, hypertensinogen [2], the question of the mechanism of enzyme action of renin has not been definitely explained. A number of facts allow us to consider renin to be a proteolytic enzyme [3]. However, up to this time no chemical characteristics of the course of this reaction have been found. Thus, it has not been possible to show that, when renin and hypertensinogen interact, an increase takes place in the free amino or carboxyl groups, or that there is an increase in nonprotein nitrogen [4, 5]; attempts to use chromatography for this purpose also have not given the desired results [5, 6].

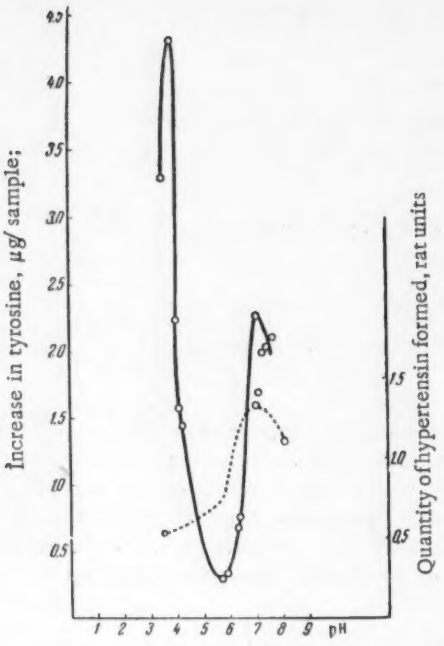
Meanwhile, the absence of biochemical methods for the determination of renin and hypertensin have made it necessary to continue the study in this direction. Because it is known that the product of the reaction between renin and hypertensinogen, namely hypertensin, contains tyrosine [7], there were grounds for expecting that the interaction of renin and hypertensinogen would be accompanied by the appearance of peptides of low molecular weight containing tyrosine. Starting from this point, in the present work we determined the quantity of tyrosine in proteinless filtrates of the incubation mixtures of renin and hypertensinogen.

METHOD

The methods of obtaining and verifying the activity of preparations of renin and hypertensinogen were specified in a previous communication [6]. Certain experiments were set up with the purer preparations of renin obtained by the method of Haas, Lamfrom and Goldblatt [8] in which renin was subjected to further purification by acetone. We used the protein fraction which precipitated at an acetone concentration between 30 and 60%. All the preparations of renin and of hypertensinogen were free from hypertensinase. We studied the action of renin on hypertensinogen at different pH values.

The experiments were carried out as follows: First we established the necessary pH value of the hypertensinogen by the addition to it of dilute solutions of HCl and NaOH. The pH was estimated by the use of a potentiometer with a calomel electrode. Then two samples were prepared: 1) experimental, with 5 to 10 ml of hypertensinogen + 1 ml of renin, and 2) control, with the same quantity of hypertensinogen. The samples were placed in a constant temperature of 38 to 40°. After two hours the proteins were precipitated by the addition of 4 volumes of 96% alcohol, after which 1 ml of renin, which had been standing in the constant-temperature oven under the same conditions, was added to the control sample. The samples were filtered and equal quantitites of the alcoholic filtrates were evaporated on a water bath. The dry residues were extracted with 12 ml of 0.3 M trichloroacetic acid. The solution was filtered and 10 ml used for determination of tyrosine content. For completeness, in experiments with purified renin extraction was done with water to which was added trichloroacetic acid until it became 0.3M.

In addition to its action on hypertensinogen, we also studied the proteolytic action of renin on denatured hemoglobin at pH 3.5 and 7.5. Two samples were prepared for this: 1) experimental: 1 ml 2% solution of hemoglobin + 0.25 ml 1.35% solution of CH_3COOH in 0.2 M $(NH_4)_2SO_4$ + 1 ml renin; 2) control: the same quantity of Hb and buffer. After 2 hours at 37°C the samples were precipitated with 0.3 M trichloroacetic acid, after which 1 ml of renin, which had been standing in the constant-temperature oven under the same conditions, was added to the control sample. The samples were filtered and 5 ml of the filtrate used for the determination of tyrosine.



Proteolytic Action of Renin on Hypertensiogen.
——— tyrosine content: --- hypertensin content.

Determination of the tyrosine content was carried out with Folin's phenol reagent [9]. The optical density was determined in a Hilger photoelectric colorimeter two minutes after adding the reagent. A 608 filter was used. The width of the cuvettes in the experiments with hypertensinogen was 5 cm, but in the hemoglobin experiments it was 1 cm.

Further, in many of the experiments we used a Beckman spectrophotometer to determine the ultraviolet absortpion coefficient at 280 mμ in the proteinless filtrates of the experimental and control samples.

RESULTS

In Table 1 are listed the data showing that when preparations of renin and hypertensinogen are incubated, an increase in tyrosine is observed in the proteinless filtrate. The quantity of tyrosine formed depends on the pH at which the reaction between renin and hypertensinogen takes place: two maxima were noted at pH 3.8 and 7. This is seen more graphically in the figure.

As seen from Table 2, when preparations of renin are incubated with hemoglobin at pH 3.5, a definite accumulation of tyrosine is observed in the proteinless filtrate. Splitting of hemoglobin in neutral solution by these preparations of renin was not observed. Analogous data were obtained spectrophotometrically.

The data listed in Table 3 show that the absorption coefficient of the experimental samples of 280 mμ is always higher than that of the control samples, which also shows the increase in the content of tyrosine, or of peptides of low molecular weight containing tyrosine, when renin is incubated with hypertensinogen (at pH 7) and with hemoglobin (at pH 3.5).

TABLE 1

Tyrosine Content of Proteinless Filtrates of Incubation Mixtures of Renin and Hypertensinogen

pH	Tyrosine content in μg/ sample		
	experiment	control	difference
3.5	17.25	13.95	+3.30
3.8	19.34	15.01	+4.33
4.0	16.41	14.18	+2.23
4.1	15.53	13.95	+1.58
4.1	14.46	13.02	+1.44
5.7	10.74	10.41	+0.33
5.7	10.88	9.98	+0.90
6.3	11.25	10.50	+0.75
6.3	12.50	11.81	+0.69
7.0	13.10	10.83	+2.28
7.0	13.95	12.36	+1.59
7.3	14.36	12.32	+2.04
7.5	13.66	11.53	+2.13

The data listed show that when preparations of renin and hypertensinogen are incubated, an increase in the tyrosine in the proteinless filtrates is observed.

A study of how this reaction depends on the pH showed the presence of two optima: at pH 3.8 (more) and at pH 7 (less), which coincides with the data shown in the literature [10]. Since, according to the data in the literature [3] and our own observations (figure), the optimum of the reaction forming hypertensin is observed at weakly alkaline pH values, one might think that the proteolytic action at acid reactions was caused not by renin, but by another proteinase. It is all the more probable that this action is nonspecific, because renin preparations in an acid medium also split hemoglobin.

The proteolytic action of preparations of renin in neutral solution have an optimum pH extremely close to the optimum pH of the reaction forming hypertensin. This action is more specific than that observed in acid medium, for hemoglobin does not split in a neutral medium. In this connection the question arises: Is it possible to ascribe the proteolytic action in a neutral medium of the prepara-

TABLE 2

Proteolytic Action of Preparations of Renin on Hemoglobin

pH	Tyrosine content in μg/sample			pH	Tyrosine content in μg/sample		
	experiment	control	difference		experiment	control	difference
3.5	22.08	17.28	+4.8	3.5	38.84	23.52	+10.32
3.5	21.96	15.12	+6.84	3.5	36.36	28.24	+ 8.12
3.5	24.0	15.96	+8.04	7.5	12.84	13.08	— 0.24
3.5	12.96	9.36	+3.6	7.5	13.32	13.68	— 0.36
3.5	15.96	11.4	+4.56	7.5	14.4	14.52	— 0.12
3.5	15.36	13.44	+1.92	7.5	21.0	21.24	— 0.27
3.5	31.92	25.32	+6.6	7.5	21.24	21.24	0
3.5	20.4	18.0	+2.4	7.5	24.24	24.84	— 0.6
3.5	19.68	17.76	+1.92	7.5	21 72	25.52	— 0.84
3.5	25.8	21.72	+4.08				

TABLE 3

UV Absorption Coefficients of the Proteinless Filtrates

Substrate	pH	Absorption coefficient at 280 mμ		
		exper.	control	difference
Hypertensinogen	7.0	0.410	0.396	+0.014
"	7.0	0.560	0.490	+0.070
"	7.0	0.502	0.450	+0.052
"	7.0	0.402	0.395	+0.007
"	7.0	0.455	0.455	0
"	7.0	0.750	0.735	+0.015
Hb	3.5	0.795	0.654	+0.141
"	3.5	0.800	0.682	+0.118
"	3.5	0.809	0.670	+0.139
"	3.5	0.785	0.580	+0.205
"	3.5	0.850	0.625	+0.125
"	3.5	0.920	0.645	+0.275
"	3.5	0.800	0.630	+0.170

tions studied to renin itself, or is this a manifestation of the action of some other proteinase which is present in the preparation as an impurity?

In order to answer this question we obtained purer preparations of renin (by the method of Haas, Lamfrom and Goldblatt, with subsequent purification by acetone). These preparations had a considerably greater (about 20 times, calculated on the basis of 1 mg of protein) capacity for forming hypertensin than the preparations used in carrying out the experiments described above. In addition, it appeared that the proteolytic activity with respect to hypertensinogen (in neutral medium) of the purer preparation was considerably lower. This shows that in the less pure preparations, in addition to renin there is present still another proteinase whose optimal pH value is close to that which is optimal for renin.

Thus, the increase in tyrosine in the proteinless filtrates in experiments with less purified preparations of renin may be attributed only partially to the proteolytic action of renin itself; evidently a considerable part of it

is due to the action of a proteinase which accompanies renin. With respect to the purer preparations of renin, it may be considered that the increase in tyrosine is caused by the proteolytic action of renin itself, for the increase in tyrosine in the experiments with these preparations (1 to 2 μg) corresponds approximately to the quantity of tyrosine to be expected, knowing the quantity of hypertensin formed in the experiment and judging by the data in the literature [7] regarding the tyrosine content of hypertensin (13%). However, to exclude definitely the presence in these preparations of a nonspecific proteinase on the basis of the experiments carried out is not considered possible. A full answer to this question requires a more detailed study of the relation between the quantity of hypertensin formed and the increase in tyrosine in experiments with large quantities of renin and of hypertensinogen of a high degree of purity.

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THE EFFECT OF ASCORBIC ACID ON THE ACTIVITY OF THE ADENOSINE TRIPHOSPHATASE OF HEART AND SKELETAL MUSCLE OF THE GUINEA PIG

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Data from the literature show that the activity of a number of enzymes depends on the degree of saturation of the organism with ascorbic acid.

Thus, in avitaminosis C there is a decrease in the activity of the amylase and esterase of the blood [1], of the cathepsin and lipase of the liver and kidneys [2], and of muscle esterase [3], while there is an increase in the activity of the proteolytic enzymes of the liver, kidneys and muscles [4]. When supplementary ascorbic acid is given to the animals there is an increase in the activity of the oxidation-reduction enzymes [5] and of catalase [6, 7].

In a number of infectious diseases, and particularly in diphtheria, along with the development of a vitamin C deficiency state, there is a disturbance of the normal contractile activity of the heart muscle. In this connection it was of interest to examine the effect of ascorbic acid on the activity of the adenosine triphosphatase (ATPase) of cardiac muscle, since the conversion of ATP, which plays a primary role in the chemical mechanism of muscular contraction, depends on the activity of this enzyme.

METHODS AND RESULTS

The activity of the ATPase of cardiac muscle of guinea pigs was determined by the amount of phosphorus splitting off from ATP under the influence of 1 mg of tissue after 30 minutes at 37° [8]. In addition to the determination of ATPase activity in cardiac muscle, the activity of this enzyme was also determined in skeletal muscle. The quantity of ascorbic acid in the cardiac and skeletal muscle was determined by the method of Birch, Harris and Ray.

The experimental animals were separated into four series. In the first series of the study, the controls, we determined the activity of the ATPase of the cardiac and skeletal muscles of guinea pigs living under the usual laboratory conditions.

From Table 1 it is seen that ATPase activity and ascorbic acid content in cardiac muscle in the control guinea pigs varied within fairly wide limits. Attention was attracted to a certain dependence of ATPase activity on the ascorbic acid content of cardiac muscle. Thus, in most of the experiments in which the ascorbic acid content of cardiac muscle was high, the ATPase activity was also noticed to be high (Experiments 7, 8 and 9), while at a low ascorbic acid content in the heart, the ATPase activity was also low (Experiments 3, 5, 6 and 10). We also observed a similar dependence of the ATPase activity on the ascorbic acid content of skeletal muscle.

In the second series of the investigation, we studied the effect of avitaminosis C on the ATPase activity of cardiac and skeletal muscle. This condition of vitamin C deficiency was brought about in the guinea pigs by giving the animals feed from which vitamin C had been removed (oats, autoclaved hay, carrots and beets). After 25 days avitaminosis C developed in the guinea pigs: the animals lost weight, became less active; autopsy showed subcutaneous hemorrhages (especially in the area of the knee joints) and hemorrhages in the muscles; the teeth were easily loosened. The data of this series are listed in Table 2, from which it is seen that in caridiac and skeletal muscle of guinea pigs on a vitamin C-deficient diet the quantity of ascorbic acid is

considerably reduced, as compared to the control data: in cardiac muscle by an average of 40%, in skeletal muscle by 37%. The ATPase activity of the heart and skeletal muscles of vitamin C-deficient animals was also noted to be lower than in control animals – in the heart by an average of 44%, in the skeletal muscles by 49%.

TABLE 1

ATPase Activity and Ascorbic Acid Content of Cardiac and Skeletal Muscles in Control Guinea Pigs

Experiment No.	Cardiac muscle		Skeletal muscle	
	ATPase activity in μg P	Quantity of ascorbic acid in mg%	ATPase activity μg P	Quantity of ascorbic acid in mg %
1	17.6	4.7	21.5	1.04
2	17.2	5.3	19.1	1.04
3	13.4	3.5	15.0	1.06
4	15.6	4.2	18.5	1.08
5	16.2	3.2	17.2	1.50
6	16.8	2.4	17.7	0.86
7	20.1	5.5	28.5	1.60
8	19.3	7.5	29.4	1.70
9	18.8	7.1	28.5	1.60
10	16.1	3.7	25.0	1.10
11	16.1	4.1	20.0	1.11
Average	17.0	4.7	21.9	1.24

TABLE 2

ATPase Activity and Ascorbic Acid Content of Cardiac and Skeletal Muscles in Vitamin C-Deficient Guinea Pigs

Duration of avitaminosis in days	Cardiac muscle		Skeletal muscle	
	ATPase activity in μg P	Quantity of ascorbic acid in mg%	ATPase activity in μg P	Quantity of ascorbic acid in mg %
17	13.5	2.2	14.0	0.86
25	9.8	3.4	8.7	0.53
28	13.7	1.7	14.0	0.72
19	9.0	2.6	13.1	0.82
32	11.8	3.6	10.3	0.80
33	10.6	3.2	9.1	0.69
29	5.8	3.1	11.0	0.90
31	5.6	2.8	8.1	0.80
33	5.8	2.2	12.8	0.85
25	10.1	3.2	11.3	0.90
Average	9.6	2.8	11.2	0.78

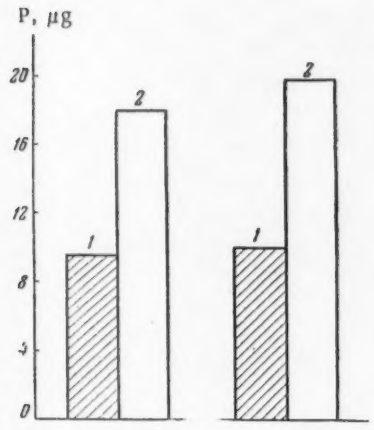
The data listed show that, together with a decrease in the quantity of ascorbic acid in the organs studied in avitaminosis C, a considerable decrease is observed in ATPase activity both of cardiac and of skeletal muscle.

In the third series of the investigation we studied the ATPase activity of cardiac and skeletal muscle when supplementary ascorbic acid was given to the guinea pigs. Ascorbic acid in the proportion of 20 mg/100 g of weight was added daily to the animal's usual feed for periods varying from 6 to 29 days in different experiments. For one day before the experiment the guinea pigs received no ascorbic acid. The results of these in-

TABLE 3

ATPase Activity of the Cardiac and Skeletal Muscles of Guinea Pigs Given Supplementary Ascorbic Acid

Experiment No.	Duration of adminstration of ascorbic acid, days	Cardiac muscle		Skeletal muscle	
		ATPase activity in μg P	Quantity of ascorbic acid in mg %	ATPase activity in μg P	Quantity of ascorbic acid in mg %
1	11	19.2	4.8	20.8	1.7
2	11	22.8	5.0	23.1	1.5
3	13	25.8	5.6	30.3	2.0
4	19	24.4	5.6	24.4	1.9
5	26	27.5	8.0	28.3	2.2
6	6	16.6	5.3	17.5	1.2
7	14	20.4	6.0	28.7	2.1
8	28	25.1	8.0	29.5	2.3
9	29	24.2	8.0	24.4	2.4
Average	–	22.9	6.2	25.2	1.9



ATPase Activity in Guinea Pigs with Avitaminosis C on Supplementary Administration of Ascorbic Acid. 1) Avitaminosis C; 2) Supplementary Administration of Ascorbic Acid.

vestigations, which are listed in Table 3, show that on supplementary adminstration to the guinea pigs of ascorbic acid, the quantity of it in the cardiac and skeletal muscle considerably exceeds that in control animals (in the heart by an average of 32%, in skeletal muscle by 53%).

The ATPase activity of the cardiac and skeletal muscle of animals which had received ascorbic acid was also higher in comparison with the ATPase activity of these tissues in control animals (in the heart by an average of 35%, in skeletal muscle by 15%). In most cases the ATPase activity is higher in tissues of those animals which had received the ascorbic acid for a greater number of days (experiments 4, 5, 8 and 9) and lower in the case of a relatively smaller number of days of administration of supplementary ascorbic acid (Experiments 1 and 6).

In the fourth series of the investigation we tried to find out whether the decrease in ATPase activity in heart and skeletal muscle of guinea pigs with avitaminosis C was an irreversible phenomenon, or whether the ATPase activity could be restored under appropriate conditions. To answer this question, the ATPase activity was determined in animals with avitaminosis C after the supplementary administration to them of ascorbic acid in a proportion of 20 mg/100 g of weight per day. The ascorbic acid was given for periods ranging from 18 to 30 days in different experiments. It should be noted that when supplementary ascorbic acid was administered to guinea pigs with avitaminosis C, the general condition of the animals improved noticeably: they became more active and gained weight.

The ascorbic acid content in the tissues studied in this series of animals was considerably higher than in guinea pigs with avitaminosis C, and amounted on the average for cardiac muscle to 5.7 mg % with variations from 3.0 to 7. 1 mg %, and for skeletal muscle it amounted to an average of 1.26 mg %, varying from 0.88 to 1.41 mg %.

The ATPase activity of cardiac muscle when supplementary ascorbic acid was fed to guinea pigs with avitaminosis C amounted on an average to 18.1 μg P, i. e. in this series of animals the activity was considerably

higher than in either animals with vitamin C deficiency or in the control guinea pigs. The ATPase activity in the skeletal muscles in this series of animals amounted to an average of 19.9 μg P, i. e. the same as in cardiac muscle; it was considerably higher than in animals with avitaminosis C and reached the level of activity of this enzyme in control guinea pigs. The data of this series of the investigation are shown in the figure.

SUMMARY

Ascorbic acid affects the activity of the ATPase both of cardiac and of skeletal muscle: in avitaminosis C, the ATPase activity of these tissues is considerably decreased, and when supplementary ascorbic acid is fed to guinea pigs ATPase activity increases. The change in ATPase activity in avitaminosis C is reversible: when supplementary ascorbic acid is fed to guinea pigs and the avitaminosis C condition disappears the ATPase activity of cardiac and skeletal muscle increases.

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AN ELECTROPHORETIC STUDY OF LIVER PROTEINS

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Notwithstanding the very large number of studies carried out in recent years on the question of changes in the protein composition of the blood serum in various physiological and especially in pathological conditions of the body, up to the present we know very little about the causes of such changes or the mechanism by which they arise.

In the work of Whipple and of a number of other authors in which a concept was worked out of dynamic equilibrium between the serum proteins and the organ proteins (especially the liver), the proposition was advocated that one of the basic causes of the changes in the ratios of the various protein fractions of the serum was a disturbance of this equilibrium. Data obtained by Kuzovleva [1] and the results of the studies by Gavrilova and Konikova [2] also confirmed this proposition. However, all these data could not serve as direct proof that disturbance of the interconversion of the proteins of the liver and of the serum is the basic factor causing changes in the ratios between the individual fractions of the serum proteins.

In order to show by direct means the possible correlation between a shift in the protein composition of the liver and other organs and changes in the protein composition of the serum, it was first necessary to get a clear conception of the protein composition of the soluble fraction of the liver.

The most frequently used method of separation of the proteins of organs, electrophoresis, is still insufficiently effective, which is explained by the excessively complex protein composition of tissue extracts as compared with blood plasma. The very large number of proteins with close electrophoretic mobility, the probable complexing of one protein with another, and, finally, the possible denaturation of the labile components in the extraction process, all lead to solid electrophorograms which are almost completely undifferentiated into peaks and which are thus made very difficult to decipher.

Thus, in order to study the interrelation between changes in the proteins of organs and of serum by the use of electrophoretic methods, the primary need was for the perfection of methods of extracting the proteins of organs and also of the method of separating them electrophoretically. The purpose of the experiments described below consisted of obtaining, on the basis of extracts prepared in a definite manner, precise electrophoretic data regarding the protein composition of the soluble fraction of the liver which could then be compared with the protein composition of the blood.

EXPERIMENTAL METHODS

The basic experiments were arranged on male white rats weighing 200 to 300 g; in each experiment the livers of several animals were taken. In addition, a series of experiments was carried out on dogs. The livers of the killed animals were perfused (in the rats, in situ; in dogs, after removal) with physiological salt solution for 3 to 5 minutes, and then immediately homogenized while being cooled.

In the first method of extraction, the homogenization took place in a glass homogenizer or a Waring blender with a quantity of physiological salt solution equal to half the weight of the liver. Small portions of ether were added to the homogenate with vigorous stirring and with cooling until it became flaky. Having be-

come very thick with increase in volume, the homogenate was centrifuged at 2500 r.p.m. with cooling. The lower transparent or slightly opalescent aqueous layer was carefully drawn off by suction, transferred to a centrifuged tube, and placed in a water bath at 37° for 15 minutes. This caused a spongy precipitate of protein to come down, which, after standing at room temperature, was separated by centrifugation at 2500 r.p.m. for 7 minutes. We obtained as a result a completely transparent solution of the color of tea, with a protein concentration of 1.7 to 2.2%. In cases in which it was desirable to extract the albumin from the liver tissue as fully as possible, we used the second method of extraction: The homogenization was carried out in a Waring blender with a fourfold volume of a mixture of equal parts of a 2% NaCl solution and butanol. The homogenate obtained was centrifuged for 25 minutes at 3000 r.p.m.; the lower layer, located beneath the "plug" of undissolved proteins, was drawn off by suction and the butanol was extracted with ether from the solution thus obtained. Since the concentration of proteins in such an extract was very low, it was necessary to make it more concentrated. For this the solution was dialyzed against distilled water, after which it was lyophilized. The dry powder thus obtained dissolved easily in veronal buffer with a pH of 8.6, so that it was possible to get a solution concentrated enough for electrophoresis. In certain experiments (with dog liver), in order to avoid lyophilization, we decreased the quantity of the 1:1 butanol-2% NaCl to half of the weight of liver. After homogenization, $\frac{1}{4}$ volume of 2% NaCl of the weight of the liver was added and the mixture again homogenized. The extract obtained after centrifugation contained 1.7 to 2.4% protein.

In some cases, for purposes of comparison, we also subjected to electrophoresis extracts obtained by the first method, but without heating. They were clarified by centrifuging at 18,000 r.p.m. for 1 hour. In some cases we studied extracts obtained by the second method, but with subsequent heating for 15 minutes at 37°. In addition, in experiments with dog liver, extracts prepared by the first and second methods were compared with extracts obtained from an acetone preparation [3] of liver by treating the dry powder with a fourfold volume of veronal buffer at pH 8.6.

Electrophoretic analysis of extracts was carried out by the method [4] which we previously used for blood serum in a Tiselius-Svensson apparatus in 0.1 N veronal buffer ($G/2 \sim 0.7$; pH 8.6) against which the extract had previously been dialyzed. The electrophoresis lasted 5 to 6 hours at a gradient of around 9 volts/cm.

RESULTS

In the extraction of proteins of the liver by physiological salt solution we usually found 40% of the total nitrogen of the liver in the centrifugate after treatment with ether. In many cases when this centrifugation was carried out for 20 minutes at 1800 r.p.m. it was possible to find 50% of the total liver nitrogen in the solution. After incubation of the solution for 15 minutes at 37° and separation of the precipitated protein, there remained in solution 35% of the total nitrogen of the homogenate in comparison with 50% before heating.

In extractions of liver with a mixture of 2% NaCl and butanol, the total quantity of protein going into the extract is considerably less than in extraction with physiological salt solution. Thus, in our experiments under these conditions, only 25% of the total liver nitrogen went over into the solution, as compared with 50% of the nitrogen going into solution in extraction by the first method. In addition, in butanol extractions (shown to be less productive) the relative quantity of albumin and of certain globulins extracted was shown to be greater than in extraction with physiological salt solution. It might be considered that, the mitochondira having been destroyed and the extraction of certain proteins made possible, especially that of albumin (Khesin [5]), the butanol prevented the solution of other liver proteins. The question of this action of butanol is of considerable interest and requires special study.

In Figure 1 are shown typical electrophorograms of protein extracts of rat liver obtained by the methods described above. In Table 1 are shown the electrophoretic mobilities of those proteins which we observed systematically in the study of extracts. As seen from Fig. 1, the most distinct electrophoretic picture was produced in the study of extracts prepared by the first method. On the electrophorogram of this extract are seen six distinct sharp peaks, one a fast one (A), then four medium zones of mobility (C, D, E, and F) and, finally, a slow peak G, not separated from the salt. If electrophoresis is carried on for a longer period, the position of the points of the peaks referred to becomes more precise and it is further possible to distinguish even more diffuse maxima, which are designated in Fig. 1b by small letters of the alphabet. In addition, in electrophorograms of rat liver it is always possible to observe one or two diffuse maxima leading peak A (mean rate, 8.5 and 6.7×10^{-5} cm^2 sec.$^{-1}$ v^{-1}).

TABLE 1

Electrophoretic Mobilities of Protein Components (in $cm^2 sec^{-1} V^{-1} \times 10^{-5}$). a) of the Soluble Fraction of Rat Liver; b) of Serum of the Rat. Figures in italics show values calculated on the basis of a small number of experiments with prolonged electrophoresis. Veronal buffer, pH 8.6; $G/2 \sim 0.07$; 0°.

Designation of fraction	A: Method of extract preparation: First with heating — Ascending boundary	A: First without heating — Ascending boundary	A: Second — Ascending boundary	Average ± standard deviation: Ascending boundary	Average ± standard deviation: Descending boundary	B: Adult animals — Ascending boundary	B: Embryos — Ascending boundary	Designation of the fraction
	7(30)*	1(4)*	6(32)*	14(66)*		17*	11*	
a_1	+	+	*8.46*					
a_2	*6.69*	+	+					Albumin
A	5.77	5.79	6.04	5.92 ± 0.22	5.62 ± 0.14	5.85 5.32	5.84 5.27	α_1-(etc.)Globulin α_1-
B_1	*4.49*	+	4.55	4.53 ± 0.18	3.96 ± 0.07		4.56	α_2- "
B_2	*4.23*	4.27	+	4.23 ± 0.05	4.06	4.16	4.22	α_3- "
C	3.65	3.66	3.61	3.63 ± 0.16	3.34 ± 0.22		3.62	α_4- "
D	3.19		3.13	3.16 ± 0.11	2.87 ± 0.20			
d	*3.06*	*3.08*				3.01		"
E	2.47	2.37	2.42	2.45 ± 0.08	2.25 ± 0.14	2.58	2.69	β_2- "
F	1.91	1.88	+	1.91 ± 0.08	1.76 ± 0.08			
f	*1.76*	+	*1.71*			1.61	1.69	γ- "
G	0.37	0.22	0	0.37	0.34			

* Number of analyses carried out; in parentheses, total number of animals.

The fact that the tips of five of the basic peaks which we observed (excluding G) did not in general lose their sharpness as the time of electrophoresis was lengthened, together with the good reproducibility of their rates from one experiment to another, gives us some basis for assuming that the velocities of corresponding boundaries reflect the electrophoretic mobilities of certain proteins which, in the given fraction, are dominant over other proteins of nearby mobilities. As far as concerns the diffuse maxima which are designated by small letters (and the number of them will evidently increase in proportion to the accumulation of further electrophoretic material as liver protein), it is more likely that their position reflects not so much the electrophoretic mobility of a specific protein as it does the result of the geometrical summation of a number of boundaries of different proteins of similar mobilities, or complexes of them, in small but comparable concentrations.

It was of interest to compare the electrophorogram just examined with the electrophoretic picture of extracts obtained by the same means, but not subjected to heating, i.e. containing all the soluble liver proteins extracted by the first method. In the electrophorogram of such an extract (Fig. 1c) there are the same more or less pronounced six maxima, as judged by their mobilities. In addition to these there is also seen a new and quite pronounced peak, B_2, which disappears almost entirely after heating the extract. This is evidently a very labile protein, precipitating easily even under such gentle action as 15-minute heating at 37°. It must be noted, however, that we were seldom able to observe such a relatively clear picture of the electrophoretic separation of the proteins of unheated extracts.*

* In most cases such a clear separation into peaks was not seen in the electrophorograms of such extracts. The smoothed-out look of the pictures obtained recalls the electrophorograms published in the work of Sorov and Cohen [6], where only high-speed centrifugation was used for the separation of soluble liver proteins. Regardless of the diffuseness of the picture B_2 was always expressed clearly enough. After warming such an extract we always obtained good electrophorograms (Figs. 1a and b).

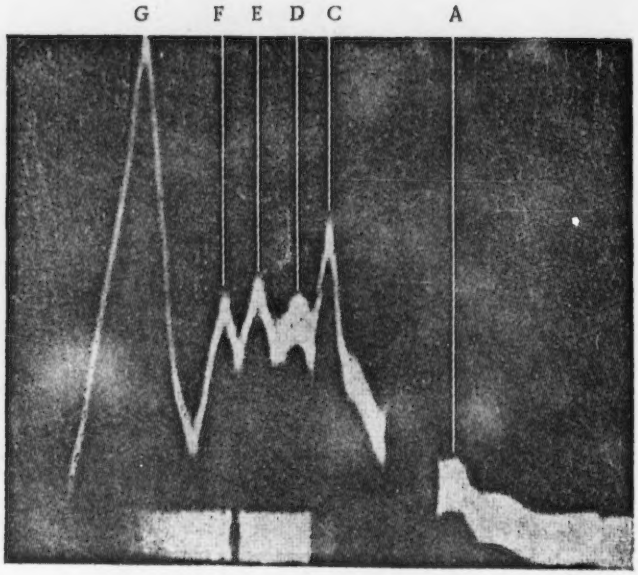

Fig. 1a

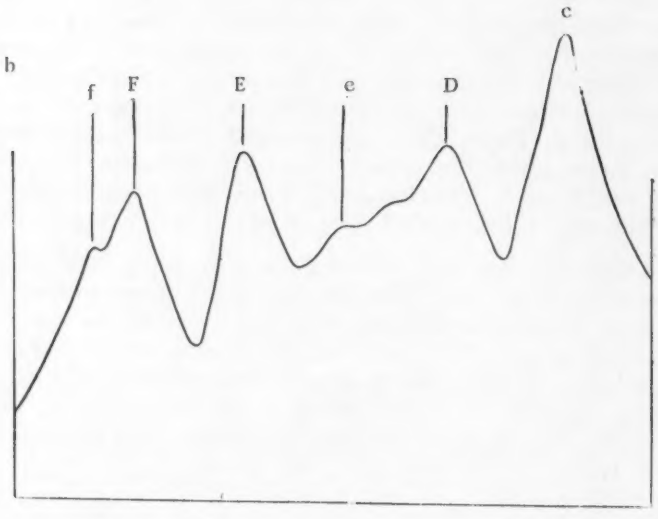

Fig. 1b

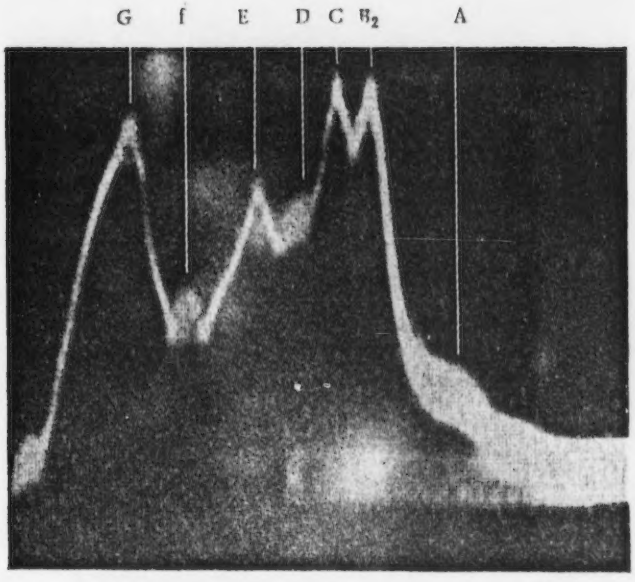

Fig. 1c

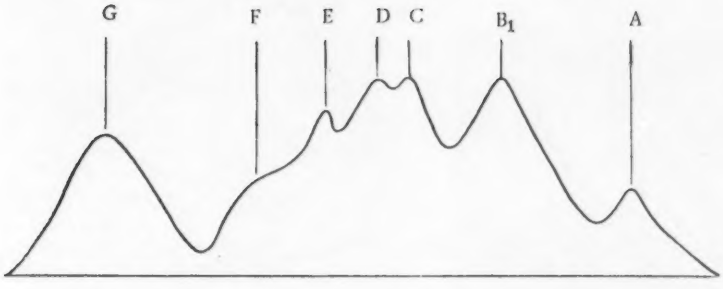

Fig. 1d

Fig. 1. Electrophorograms of the soluble fraction of the liver of the rat (ascending boundary): a) Extract prepared by the first method; duration of electrophoresis, 2 hours 30 minutes. b) Same experiment; duration of electrophoresis, 5 hours 49 minutes. c) Extract prepared by the first method without heating to 37°; duration of electrophoresis, 2 hours 6 minutes. d) Extract prepared by the second method; duration of electrophoresis, 3 hours 18 minutes.

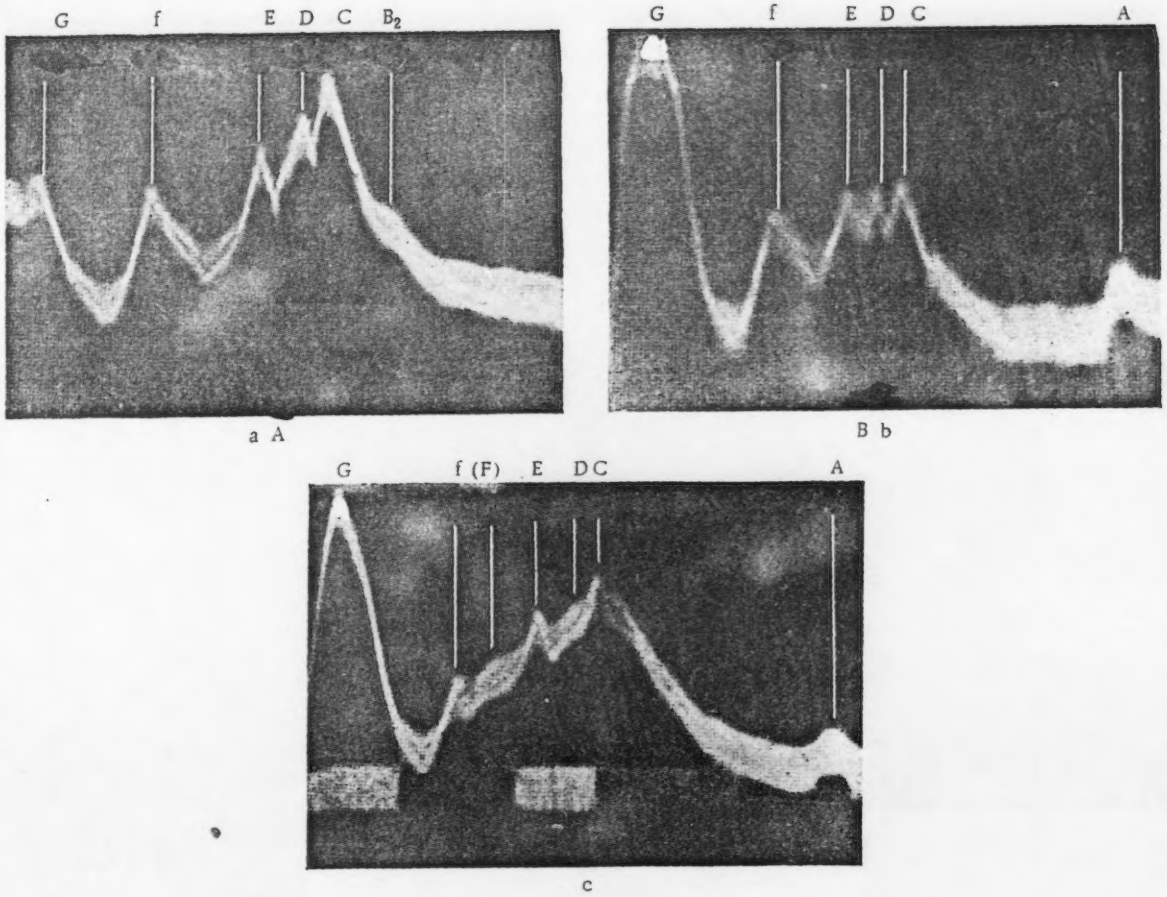

Fig. 2. Electrophorgrams of the soluble fraction of dog liver (ascending boundary):
a) Extract prepared by the first method; duration of electrophoresis, 3 hours 55 minutes. b) Extract prepared by the second method and then heated to 37°; duration of electrophoresis, 3 hours 1 minute. c) solution of acetonized preparation; duration of electrophoresis, exactly 3 hours.

TABLE 2

Relative Content of Electrophoretic Fractions in Extracts of Rat Liver (a) and Serum (b)

Name of fraction	Method of preparing extract			%	Name of fraction
	First method with heating	First method without heating	Second Method		
$a_1 + a_2$	1.1	2.0	2.3		Albumin
A	5.5	7.2	9.4	49.0	α_1 [etc.] globulin
				9.3	α_1- "
B_1	13.6	20.8	23.9	0.6	α_2- "
B_2	15.7	17.0	11.4	9.4	α_3- "
C	10.0	10.2		8.5	α_4- "
D + d	15.8	11.4	37.9	12.4	β_1- "
E	9.0	6.6			β_2- "
F	3.4			10.8	γ- "
f	25.9	24.8	15.1		
G*					

* The area of peak G runs together on the electrophorograms with the area of the salt peak. To introduce a correction into the latter area it was determined by calculation, proceeding from the ratio of the area of peak G to the ascending and descending curves. On the average, the salt peak thus calculated constituted about 12% of the total area of the electrophorogram.

Going over to the examination of the curves obtained by electrophoresis of liver proteins extracted with butanol, it must first be stated that all the basic peaks which we observed on electrophoresis of warmed liver extract were present also in these electrophorograms (Fig. 1d). As a rule, peak A was more prominent in them, and in addition a distinct new peak was seen in these electrophorograms (B_1).

At the present time it is still difficult to say what the appearance of peak B_1 is related to. It is possible that this protein fraction is contained in the cytoplasmic granules and emerges into the solution when they are destroyed by butanol.

Thus, there enter into the composition of the soluble fraction of rat liver no less than eight separate protein components which predominate over a background consisting, evidently, of a multiplicity of proteins poorly separable into discrete fractions. Data relative to quantitative ratios of separate fractions of soluble rat-liver proteins are shown in Table 2.

As seen from the electrophorograms, the values listed in the table may have only an approximate and, to a certain degree, a limited significance, since the actual content of the protein component which, strictly speaking, determines the mobility of this fraction, will inevitably be made larger at the expense of other proteins with closely similar mobilities. Nevertheless, these data may still be used for purposes of rough comparison.

In Fig. 2 are shown examples of electrophorograms of dog-liver extracts; the corresponding electrophoretic mobilities of the peaks observed are shown in Table 3. Although we studied the soluble proteins of dog liver less carefully (a total of 9 electrophoretic analyses were carried out using the livers from 4 dogs) the data obtained proved to be adequate to fix the characteristics of a resemblance in protein composition between the soluble fractions of the liver of the dog and the rat.

From Table 3 it is clearly seen that 5 sharp peaks, designated, in analogy with rat liver, A, C, D, E, and f, are present in the electrophorograms of all extracts obtained by various extraction methods.

These very stable protein components of the soluble fraction of dog liver stand out most distinctly of all, just as in the case of the rat, in extracts prepared by the first method after removal of the thermolabile proteins. In extracts not subjected to heating, in addition to the only weakly distinguishable first five fractions, there is present a clear sixth peak, B_2.

TABLE 3

Electrophoretic Mobilities of the Protein Components (in $cm^2 sec^{-1} V^{-1} \times 10^{-5}$); a) Soluble fraction of dog liver (ascending boundary); b) Dog serum [4]. Veronal buffer, pH 8.6; G/2 ~ 0.07; 0°.

a*

Method of preparation of extract	No. of dogs	Fraction														
		a_1	a_2	A	b_1	b_2	B_1	B_2	c	C	D	e	E	F	f	G
First, with heating	2	—	—	6.95	—	*5.65*	*5.12*	*4.33*	—	3.54	3.24	*3.00*	2.79	—	1.38	+
First, without heating	2	—	—	7.05	—	*5.91*	—	4.33	*3.96*	*3.66*	*3.35*	*3.13*	*2.92*	*1.80*	1.50	+
	3	*10.2—9.0*	*7.57*	7.14	—	*5.45*	—	4.63	*4.12*	—	—	*3.10*	—	*1.97*	*1.61*	+
	4	—	—	7.13	—	—	—	4.38	—	3.88	3.29	—	2.76	*1.85*	*1.56*	+
Second, with heating	2	*12.4—10.2*	*7.91*	7.23	—	*5.90*	—	—	—	3.55	3.24	—	2.79	—	1.51	+
Second, without heating	2	*10.6—9.5*	—	7.40	6.18	*5.47*	*5.10*	—	—	*3.73*	3.19	*2.85*	2.57	—	1.64	+
	1	*9.5*	*7.90*	6.97	—	—	—	—	—	3.44	—	—	2.77	1.98	*1.55*	+
Acetonized preparation	2	*10.8*	—	7.63	*6.28*	*5.68*	*4.74*	—	*4.09*	3.76	*3.40*	—	2.79	*2.15*	1.54	+
	1	*10.5*	*7.83*	6.79	—	—	*4.90*	4.58	—	3.67	—	—	2.92	*2.02*	—	+
Average	—	*12.4—9.0*	*7.80*	7.15	*6.23*	*5.67*	*4.96*	4.45	*4.06*	3.65	3.28	*3.02*	2.79	*1.96*	1.54	+

b

Main fractions	Albumin	Globulin									
		α_1	α_2	α_3		α_4		β_1		β_2	γ	
Mobilities (ascending boundary)	7.00	6.35	5.48	4.82		4.16		3.24		2.48	1.53	
Subfraction	—	—	—	α_3'	α_3''	α_4'	α_4''	β_1'	β_1''	—	γ'	γ''
Mobilities (ascending boundary)	—	—	—	4.98	4.65	4.16	3.87	3.47	3.10	—	1.83	1.41

* The electrophoretic mobilities of the peaks are shown separately for each experiments; the mobilities of the sharp, well-expressed peaks are shown in ordinary figures, while the orienting mobilities of the diffuse maxima are shown in italics.

Thus, the six established fractions of the soluble protein of the liver of the dog are similar to the same fractions of rat liver, with respect both to the relation to certain physico-chemical factors of the action of extraction, and also to subsequent processing of the extracts.

In these experiments with the liver of the dog we did not succeed in getting a definite peak corresponding in its position on the electrophorogram to peak B_1 in the butanol extract of rat liver.

In addition to the fraction just referred to in extracts of the liver of the dog and also of the rat, there was always present in abundance an only slightly mobile fraction, G, but the electrophoretic heterogeneity of this fraction in experiments with the liver of the dog appeared more distinctly than in experiments with rat liver. Sometimes we observed in this fraction under certain conditions of electrophoresis (pH 8.6) a subfraction exhibiting weak mobility toward the cathode.

In connection with the question of the general setup at the beginning, it was of interest to compare the mobilities of the protein fractions of the liver with the mobilities of the protein fractions of the serum. For this purpose we have shown in Tables 1, 2 and 3 data on the electrophoretic analysis of the serum of rats (of grown animals and of embryos [7]), and of dogs [4], which we previously obtained under the same conditions of electrophoresis.

Comparing the electrophoretic mobilities of the basic protein components of the soluble fraction of the liver with the protein fractions of the serum, one may be convinced that both in rats and in dogs the mobility of liver protein fraction A corresponds practically, within the limits of error of the method, to the mobility of the serum albumin. This gives us a basis for assuming that this fraction represents albumin synthesized in the liver and then transferred to the blood. Electrophoretic analysis does not permit us to decide the question: Is there some other albumin in the liver which is distinct from the albumin of the blood serum? This question requires a special investigation which is being undertaken at the present time in our laboratory.

The mobilities of the liver protein fractions B_1, B_2, and C lie in the mobility zone of the α-fraction of the serum protein, while in the rat these liver protein fractions coincide in mobility with the protein fractions of embryonal serum, we regret that we do not have at our disposal corresponding data for embryonal blood in the dog. The fact that the mobilities of specific embryonal protein fractions of the rat, α_2 and α_4-globulins, are identical with the mobilities of the B_1- and C-fractions of the liver protein of adult animals may show the origin in the liver of these embryonal protein fractions of the serum.

We did not observe any coincidence of mobilities on comparing protein components of the liver with the α-globulin serum fractions of adult animals. It may be noted that in rats, the liver protein fraction B_2 has a mobility close to that of the α_3-globulin fraction of the blood, but in the dog B_2 lags even somewhat behind the slow serum subfraction α_3''-globulin, while the following C-fraction of liver protein occupies a position between the slow subfraction α_4''-globulin and the fast subfraction β_1'-globulin of the serum. Liver protein fraction D has a mobility close to that of β_1-globulin of the serum: in the dog the mobility of these protein components of the liver and serum practically coincide, while in the rat the liver protein component leads the serum β_1-globulin somewhat in the electric field.

The liver protein E fraction lies in the same zone as the β_2-globulin of the blood, but does not coincide with it. Finally, the liver F fraction corresponds in its mobility zone with the γ-globulin of the blood: in the dog, the coincidence of the f-protein fraction in mean mobility with the γ-globulin of the serum is fairly close.

Inasmuch as the literature of recent years contains many references to the fact that a leading albumin fraction may be observed in the serum, the question arises as to whether it is identical with the fast-moving fractions of the liver, and also the question as to whether they represent proteins or big polypeptides.

On comparing the relative quantities of the protein components of extracts of the liver and blood serum which are close in mobility (for data for rats, see Table 2), it must be noted that the relative content of fraction A is several times lower than the content of serum albumin. Even in extracts obtained by the use of butanol, which made possible the complete extraction of albumin from the mitochondria, the fraction A content amounted to only 9.4% of the total quantity of protein in the extracts, in comparison with the 49% albumin content in the serum.

The relative content of the sum of the B_1 and B_2 protein fractions of the liver in extracts which are rich in them, i.e. obtained by the first method without heating and by the second method, on the other hand, is considerably greater than the content of serum proteins corresponding to this region of mobility (20 to 25%

against 10% of the relative quantities of α_2- and α_3-globulins of the serum. To the same degree this applies to fraction C, representing 15 to 17% of liver extracts, while in the blood of adult rats, the proteins corresponding to the same mobility zone (α_4-globulins) are, in general, not present in noticeable quantities.

The relative quantities of the D and E fractions of liver proved to be of the same order of magnitude as those of the β_1 and β_2-globulins of the blood which correspond to them in mobility. The same may be said of F-fractions of the liver and of the γ-globulins of the blood.

In liver extracts, a prominent feature is the comparatively high—about 25%—content of the only slightly mobile fraction G, which evidently does not have an analog in the blood on the basis of behavior in an electric field. Further detailed study is required for the question of the proteins entering into the composition of this fraction.

The data which we obtained relative to the protein composition of the fractions of the soluble proteins of the liver coincides well in some respects with the data in the literature.

In the work of Sorov and Cohen [6] which has already been mentioned, when soluble liver proteins of the rat underwent electrophoresis under circumstances analogous to ours, the presence was established in extracts of nine separate fractions; however, most of the maxima on the electrophorograms presented by these authors were not distinct. Nevertheless it must be mentioned that for a number of peaks the mobility values obtained by Sorov and Cohen and by us coincided well. Thus, for example, the mobility of the peak of albumins, according to Sorov and Cohen's data, is 5.71×10^{-5} cm^2 sec^{-1} V^{-1} while according to our data it is 5.92×10^{-5} cm^2 sec^{-1} V^{-1}.

The data which we obtained differ sharply from the data of De Lamirande and Allard [8]. These authors studied electrophoretically the protein composition of extracts of the nuclear, mitochondrial and hyaloplasm fractions of rat liver and then calculated the total content of electrophoretic fractions in relation to the total quantity of soluble proteins in liver homogenate. According to their data, in the soluble proteins of the liver may be observed six electrophoretic fractions, three of which move more rapidly than albumin. The quantity of these fast-moving fractions makes up, according to the authors' data, about 60% of all the soluble proteins of the liver. In addition to these fast-moving proteins, these authors found a fraction with a mobility approximately corresponding to our fraction C, and two slowly-moving fractions, one of which is close to our fraction F, while the other is close to one of the fractions found by Sorov and Cohen. It must be noted that, according to these authors' data, proteins with the mobility of serum albumin are completely absent from the liver, nor are there in the liver proteins corresponding to the serum α-globulin. They did not observe peak G with minimum mobility, the presence of which was established by us and by other authors. With respect to fractions with a migration rate faster than albumin, some of these were observed both on our electrophorograms and on those of Sorov and Cohen, but the quantity of these proteins amounted to not more than 5%, and not 60% as mentioned by De Lamirande, et al. Without special verification it is difficult to say what the discovery of such a large quantity of fast-moving protein, which nobody has confirmed, is connected with. We will only say that we have studied the fresh hyaloplasm of the rat and did not discover in it a large quantity of fast-moving protein. As is seen in other work in the literature on the electrophoresis of liver proteins using mobile boundaries, either a very small number of fractions was found as by Hoffman and Schectman [9], or the electrophoresis was carried out under completely different conditions as in the work of Eldredge and Luck [10]; therefore these results are in no way to be compared with ours.

As shown by our data and those of the literature, a comparison of the soluble fractions of the liver and blood serum from the point of view of the relative quantity in their composition of proteins with the mobility of albumin and of the proteins of the zone of mobility of the slow subfractions of the α-globulins and β-globulins of the blood suggests a sharp distinction in protein composition between the liver and the blood. If, in the blood, the albumin content amounts to about 50%, while the slow α and β-globulins are about 30%, in the soluble fractions of the liver the reverse ratio is seen: the proteins corresponding in mobility to serum albumin constitute not more than 10%, while those corresponding to the slow α and β-globulins of the serum are not less than 50%.

On the basis of these data it may be concluded that a disturbance of protein metabolism related to a more or less considerable transfer of liver protein to the blood should be accompanied by a clear decrease in proteins

with the mobility of albumin and a noticeable increase in the serum content of proteins with the mobility of α and β-globulins. Such changes are known to be observed in many diseases. It is thus possible to suppose that the cause of these changes consists of an increase in the transfer of liver proteins into the blood. To verify this assumption we carried out special experiments using electrophoresis on paper.

SUMMARY

In the electrophoretic analysis of the proteins of the liver of the rat, using the Tiselius-Svensson apparatus, it is possible to discover up to 8 fundamental protein fractions and, in addition, a number of less definite fractions. The presence or absence in the solution of certain of these fractions depends to a known degree on the method of extraction of the liver proteins and further on how the extracts are processed.

On extraction of the proteins with 0.85% sodium chloride and subsequent treatment with either and with removal from the solution obtained of a part of the proteins by heating at 37° for 15 minutes (first method), there is produced a well-resolved electrophorogram on which six separate fractions are indicated. The mobilities of five of these correspond with respect to the mobility zone to albumin, β and γ-globulins of the rat, while the mobility of the sixth peak is close to zero (at pH 8.6). On electrophoretic analysis of the same extract without previous heating, one more peak is seen, with a mobility lying within the limits of the α-globulins of the blood serum. On extraction of the liver proteins by a 1:1 mixture of 2% NaCl and butanol (second method) it is possible to distinguish one more (an eighth) fraction the mobility of which corresponds to serum α-globulins.

With respect to relative quantity, the basic group of proteins in rat-liver extracts consists of proteins corresponding in mobility zone to the slow α-globulins of the serum (30 to 35%), β and γ-globulins about 30% and the still slower proteins, about 25%. In the portion of the fraction corresponding to serum albumin is found only 10 to 15% of the total extracted protein.

On the electrophorograms of extracts of dog liver obtained by the first method, the same six basic fractions were also discovered as in protein extracts of rat liver.

In extractions made by the first method but without further heating, and in those made by the second method, there are seen in the solution supplementary peaks with a mobility close to that of the α-globulins of dog serum.

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THE MECHANISM OF ACTION OF CADMIUM IONS ON THE CONTRACTION OF CARDIAC MUSCLE

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By now it has been established that the inhibitory action of salts of certain heavy metals on many physiological and biological processes is caused by the ability of these substances to react with sulfhydryl groups, which are exceedingly important in tissue metabolism [1, 2].

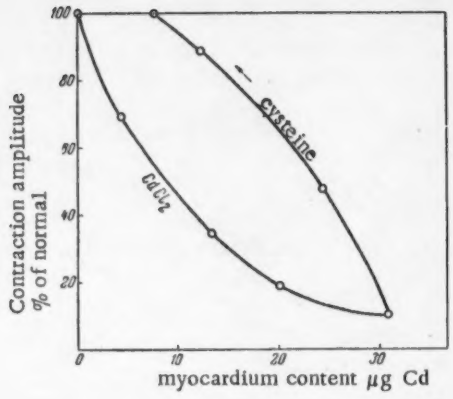
By the use of salts of silver, mercury and cadmium, the important part played by SH groups in the contraction of caridac muscle has been shown. $AgNO_3$, $HgCl_2$ and $CdCl_2$ suppress, while cysteine or 2, 3-dimercaptopropanol restore, the amplitude of contraction of the isolated ventricle of the frog's heart [3, 4]. In experiments with radioactive isotopes of silver ($Ag^{110}NO_3$) and mercury ($Hg^{203}Cl_2$), it was established that the inhibition of the contractile property of cardiac muscle takes place at an accumulation of 20+ μg of Ag or 250+ μg of Hg per g of tissue. Cysteine completely restores the function disturbed by silver or mercury, uniting with that part of the heavy metal accumulated by the tissue structures [5-7].

In the present work are shown certain new data obtained in experiments with radioactive cadmium ($Cd^{115}Cl_2$), which illustrate the great significance of the tissue thiol groups in the contractile process of heart muscle.

The experiments were carried out on the isolated ventricle of the frog's heart which was contracting from single stimuli by an induction coil in a rhythm of 30 contractions per minute. The amplitude of the contractions was recorded mechanically on a kymograph. In the experiments we used Ringer's solution in which was dissolved $Cd^{115}Cl_2$ at a concentration of 1×10^{-4} g/ml and a specific activity of 2.7×10^{-2} microcuries/ml. The solution was introduced into the cavity of the ventricle through a glass cannula. After each administration of $C^{115}Cl_2$, the ventricle was carefully washed to remove the Cd^{115} not combined with the tissue. The relative quantity of Cd^{115} accumulated by the heart muscle was determined by the intensity of emission of the Cd^{115} which was done by a Geiger-Mueller unit, the counter tube of which was placed at a distance of 1 to 1.5 cm from the ventricle. Calculation of the absolute quantity of cadmium in the heart muscle was carried out at the end of the experiment by comparison of the radioactivity of the heart, suspended on the cannula, with a tissue homogenate prepared from this heart, and with the intensity of emission of a standard solution of $Cd^{115}Cl_2$.

In the first series of experiments we studied the dependence of the inhibiting effect of the action of $CdCl_2$ on the quantity of Cd accumulated by the heart muscle. The $Cd^{115}Cl_2$ was introduced into the cannula for 1 minute. After carefully washing out the heart with Ringer's solution, we recorded the amplitude of the contraction of the myocardium and determined the intensity of emission of the cadmium accumulated by the cardiac muscle.

In the figure is shown a graph of typical experiment of this series from which it is seen that as the cadmium accumulates in the cardiac muscle, the amplitude of the contractions of the ventricle decreases. Complete inhibition of the cardiac contractions usually begins at an accumulation of 30 to 40 or more μg of Cd per g of tissue. Under the action of cysteine at a dilution of 1×10^{-4}, simultaneously with a decrease in the quantity of cadmium in the tissue, the amplitude of the cardiac contractions is restored. It must be noted that cysteine does



Variations of contractile properties of the cardiac muscle (isotonic contractions) under action of $Cd^{115}Cl_2$ and cysteine plotted against amounts of Cd^{115} accumulation in myocardium.

not remove the cadmium completely from its state of combination with the tissues. Even after prolonged action of a cysteine solution, when the amplitude is fully restored up to 15 to 30% of the original quantity of Cd usually remains in the cardiac muscle. Urea and Ringer's solution without potassium ions, which are known to restore temporarily the amplitude of contraction of heart muscle [3, 8], as well as the usual Ringer's solution, produce almost no change in the Cd content of the ventricle.

In the following series of experiments we determined the quantity of free SH groups the blocking of which leads to suppression of the contractile properties of the cardiac muscle. These experiments were carried out by a method [6] based on the following principle: A comparison is made of the quantity of Cd^{115} required to produce complete inhibition of the contractile properties of cardiac muscle with the quantity of Cd^{115} which must be added to the homogenate of the ventricle to block all free SH groups. The experiments were carried out as follows: A solution of $Cd^{115}Cl_2$ diluted 1×10^{-4} with a specific activity of 2.7×10^{-2} μc/ml was introduced for 2 minutes through the cannula. Complete suppression of contraction of the heart muscle began. After careful washing out of the heart by Ringer's solution, the ventricle was removed from the cannula, ground in mortar, and the resulting brei was diluted by Ringer's solution 1:5. The homogenate produced was transferred in 0.05 ml quantities by micropipette to glass slides with a depression. Then to each sample were added the following reagents (in ml):

Name of reagent	Glass slide no.				
	1	2	3	4	5
$Cd^{115}Cl_2$ at a concentration of 1×10^{-3} g/ml and a specific activity of 0.25 μc/ml	–	0.02	0.04	0.05	0.07
H_2O	0.07	0.05	0.03	0.02	–
8% sodium nitroprusside	0.02	0.02	0.02	0.02	0.02
20% NH_4OH	0.02	0.02	0.02	0.02	0.02

In samples containing free SH groups, a red color appeared. The nitroprusside test was negative, as a rule, in the fourth sample (rarely in the fifth), i.e. when 0.24 mg Cd was added to 1 g of tissue. Then in the first sample and in the sample with a negative nitroprusside test, the intensity of emission of the Cd^{115} was determined.

From the data of the six experiments listed in Table 1, it is seen that the ventricle of the frog's heart loses its contractile properties on accumulation within the cardiac muscle of cadmium amounting to 0.35 to 1.2% of the maximum quantity of cadmium which, when added to the tissue, completely blocks all free sulfhydryl groups. Thus, blockage by cadmium of more than 0.35% of the free SH groups of the ventricle produces complete suppression of contraction of the myocardium.

The data obtained raise the interesting question: With which proteins of the tissue does cadmium react? Experiments in this direction were carried out in the following manner:

The ventricle, treated with $Cd^{115}Cl_2$ at a dilution of 1×10^{-4} with a specific activity of 2.5×10^{-2} μc/ml, was ground in a mortar and the resulting brei diluted 1:100 with distilled water. After the intensity of emission of the homogenate had been determined, the homogenate was fractionated by the usual method with Weber's solution and water into salt- and water-soluble proteins and an insoluble remainder, and the distribution of Cd^{115} in these fractions was determined.

TABLE 1

Cd^{115} Content in Ventricle Homogenate after Perfusion of the Heart with a Solution of $Cd^{115}Cl_2$ and with Complete Inactivation of Free SH Groups

Quantity of Cd^{115} (in impulses/ min.) in 10 mg of homogenate (I)	Quantity of Cd^{115} (in impulses/ / min.), the addition of which to 10 mg of homogenate blocks the Sh groups (II)	I/II, %
61	8293	0.75
49	9438	0.52
90	11 582	0.78
150	12 797	1.16
67	8975	0.75
29	8302	0.35

Table 2 contains the data from 7 experiments showing the Cd^{115} content (in impulses per minute) in all homogenates of the ventricle and various fractions of it. It was shown that about 80% of the cadmium is found in the insoluble remainder and only about 20% of the cadmium is in the water – and salt-soluble fractions of the proteins. In experiments with the fractionation of homogenates of a ventricle the contractile property of which had been first suppressed by cadmium and then restored by cysteine, we were able to determine that the decrease in Cd^{115} in the ventricle takes place principally at the expense of a decrease in the quantity of it in the insoluble remainder.

The data listed show that the tissue thiol compounds play an important role in the act of contraction of the heart muscle. Blockade of the sulfhydryl groups of these compounds by the thiol poison, $CdCl_2$, leads to suppression of the amplitude of contraction of the cardiac muscle, while cysteine restores the contractile property of the myocardium. Complete inhibition of contraction takes place with an accumulation of 30 to 40 μg Cd per g of tissue, when 0.5 to 2.0% of the free tissue SH groups are blocked.

TABLE 2

Quantity of Cd^{115} (in impulses/ min.) in Ventricle Homogenates and in Various Fractions of Cardiac Muscle

Fraction	Expt. No.							Average in %
	1	2	3	4	5	6	7	
Homogenate	159	230	195	250	450	344	300	100
Water-soluble fraction	27.0	30.7	25.2	27.5	41.5	41.0	24.0	
Ditto, %	17.0	13.4	12.9	10.8	9.2	11.9	8.0	12.5
Salt-soluble fraction	11.2	17.0	11.5	20.0	16.2	18.0	10.0	
Ditto, %	7.0	7.4	6.3	8.0	3.7	5.2	3.3	5.7
Insoluble remainder	127.0	179.0	120.0	186.0	393.0	285.0	270.0	
Remainder, %	80.0	78.0	62.0	75.0	87.2	83.0	90.0	79.2

We obtained analogous data in other work in experiments with $AgNO_3$. Blockade of about 1% of the free SH groups of the ventricle of the heart of the frog by this thiol poison also leads to disturbance of the act of contraction [7]. However, in distinction to the extremely rapid action of $CdCl_2$, developing directly at the time of contact of the poison with the tissues, the effect of the action of Ag ions develops slowly, in many cases after the $AgNO_3$ has been washed out of the cavity of the ventricle. Under the influence of cadmium, what takes place is only suppression of the amplitude of contraction of the myocardium, while tetany develops under the action of silver. These divergences in the phenomena resulting from poisoning of the heart muscle by salts of Ag and Cd are the result of a difference in the manner of action of both substances on the cell. Apparently silver, like the other thiol poison monoiodoacetate, acts on that aspect of the metabolism of the muscles which is connected with the energy relations involved in contraction, blocking the tissue glycolytic processes leading to contraction – the Lundsgaard effect [9]. The more rapid appearance of the cadmium effect shows the action of Cd ions on the surface structures of the muscle fiber, possibly by disturbing the permeability of the cell membrane, which is known to be dependent on SH groups [10]. That the action of cadmium is evidently mostly on the surface structure of the

cell is suggested by the discovery of Cd^{115} principally in the insoluble remainder of muscle, a considerable part of which is constituted by cell membrane structures.

SUMMARY

Complete suppression of the contractile properties of the ventricle of the frog heart under the action of Cd ions begins at an accumulation of 30 to 40 μg Cd per gram of tissue. This quantity of Cd blocks 0.35% and more of the free tissue SH groups. The restoration of the contractile properties of the myocardium under the action of cysteine is accompanied by a decrease in the quantity of Cd in the heart muscle.

Experiments with the fractionation of proteins of the cardiac muscle showed that the greater part of the Cd is combined with the remainder not soluble in water or salt solution.

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STUDIES OF NUCLEOPROTEINS. RELATION OF THE ACTIVITY OF ALKALINE PHOSPHATASE TO THE CONTRACTILE PROPERTIES AND STRUCTURAL STATE OF DESOXYRIBONUCLEOPROTEINS

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In the course of the past decade, numerous proofs have been obtained of the exceptional importance of nucleoproteins in the metabolic processes related to the specific synthesis of protein.

In the multiplicity of works referring to the active participation of nucleoproteins in the metabolic processes, the communications appearing during recent years concerning the possible catalytic functions of these compounds have been of considerable interest. Presnov [1], studying the distribution of phosphatase in normal and cancerous tissue, directed attention to the phosphatase activity of the chromatin masses consisting of desoxyribonucleoprotein, and to the redistribution of alkaline phosphatase in the fixation of tissues, which also corresponds to the nature of the change in the redistribution of the nucleoprotein of the nucleus when the cell is damaged. Beginning with these observations, the author designed experiments for the study of the glycerophosphatase activity of desoxyribonucleoproteins isolated from calf thymus by Mirsky and Pollister's method [2] and came to the conclusion that desoxyribonucleoprotein (DNP) is an enzyme having glycerophosphatase activity with an optimum of action at pH 9.0 (alkaline phosphatase) and at pH 5.0 (acid phosphatase). Nucleoprotein did not lose phosphatase activity when salted out many times (up to 11 times), on dialysis, etc. The catalytic activity of DNP is inhibited by enzyme poisons and on heating to 70°.

Stern, et al.,[3] studied the catalytic activity of desoxyribonucleoproteins extracted from calf thymus [4] with reference to various phosphorus-containing substrates. It was established that ATP and ADP are acted on to a considerable extent; other compounds are affected only weakly (hexose phosphates; glycerophosphate, etc.) or are not acted on at all (pyrophosphate, phytate). The experiments undertaken by the authors to isolate the enzymatic activity factor from nucleoprotein appeared to be without success.

Shapot and Nemchinskaya [5] found no ATPase activity in DNP.

The contradictory data in the works cited may evidently be explained by differences in the methods of isolation of DNP and of the use of desoxyribonuclease inhibitors; with regard to the latter, there is in the work of Stern [3] a direct statement that when use was made of arsenate or citrate (which had just been used by Shapot and Nemchinskaya) as inhibitors of the depolymerizing action, no enzymatic activity of DNP with respect to ATP was discovered.

There is in the literature a communication dealing with the enzymatic activity of ribo- and desoxyribonucleates. Hurst et al. [6], studying the composition of mononucleotides of desoxyribonucleic acids, discovered that certain types of desoxyribonucleates, especially those obtained from the liver, showed considerable phosphomonoesterase activity. Enzymatic activity was not eliminated on deproteinization. Binkley [7] observed hydrolytic action by preparations of DNA and RNA.

All these works show possible enzymatic activity of nucleoproteins. The observed relation of phosphatase activity to desoxyribonucleoproteins agrees with the abundant data from histochemical investigations into the localization of the phosphatase activity in nuclei isolated from various tissues, and of other histochemical investigations into the high phosphatase activity of the chromosomes [8-12].

Certain authors, referring to the identical localization of DNA and alkaline phosphatase in the chromosomes, have discussed the question of the special function of the latter in the metabolism of nucleic acids [13]. Brachet and Jeener [14] postulate the existence of a relation between phosphatase activity and the phosphorus metabolism of DNA.

At the present time there is still no apparent solution to the question of how the enzyme phosphatase is related to nucleic acid; we do not know whether the observed phosphatase activity of desoxyribonucleoproteins isolated from the tissue is a result of the adsorption of the enzyme on the complex or an artificial combination of the protein components in the process of extraction of nucleoproteins, or whether this bond is preformed in the cell.

There are numerous data in the literature referring to the dissociation of nucleoproteins on extraction by 1 M NaCl [15] and to the change in protein composition of the complex in the process of purification [16]. Shapot and Nemchinskaya [5]refer to the possibility of a stable, absorption type of bond between the enzymes and the nucleoprotein complex.

To answer the question of the interrelation of the phosphatase enzyme with desoxyribonucleoprotein, we studied the manner in which the phosphatase activity depends on changes in the molecular-structural condition of the nucleoprotein. We investigated the effect of chemical agents and of physico-mechanical action on the contractile properties and alkaline phosphatase activity of the complex. For chemical action, we used various concentrations of Ca or Mg ions showing a specific effect both on the alkaline phosphatase activity and on the contractile properties of nucleoproteins.

METHOD

Obtaining Desoxyribonucleoproteins

Preparations of DNP were obtained from calf thymus and from the liver of large horned cattle by the method of Mirsky and Pollister which we changed somewhat to correspond to the modification introduced into this method by Lak [17]. These changes consisted in the substitution for the physiological salt solution used for the first tissue extraction of 0.4 M NaCl, which affords fuller and quicker extraction of cytoplasmic proteins. For the second extraction, physiological salt solution was used. Sodium citrate in a concentration of 0.01 M was added to the solution as a desoxyribonuclease inhibitor. Further processing was carried out as described in the method. The tissue remaining after separation of the cytoplasmic proteins was extracted at length with 1 M NaCl (for 3 to 4 days) until a viscous solution was produced; the remaining insoluble tissue was removed by centrifugation (1800 g). We purified the nucleoproteins obtained by precipitating them out many times (up to 8 to 10 times) by dilution of the solution with six times its volume of water. All work was carried out at a temperature of 2 to 4°.

In the DNP solutions we determined the N content by Conway's method after adding H_2SO_4, and the total phosphorus by the method of Fiske and Subbarow. The concentration of DNP in the solutions amounted to 0.3 to 0.5 mg N/ml. The N/P ratio in thymus preparations was 3.6 to 3.7; in liver preparations it was a little higher (3.8 to 3.9).

Substrate. The catalytic activity of the preparations was determined with respect to sodium α, β-glycerophosphate in a concentration corresponding to a P content of 0.25 mg P/ml.

Determination of Phosphatase Activity

The alkaline phosphatase activity was determined at pH 9.4 with medinal (sodium barbital) or borate buffer. Two ml of a salt solution of DNP were poured into 12 ml of cooled, distilled water; on cautious stirring with a rod the molecules of nucleoprotein became oriented in the direction of motion of the solution and formed gelatinous threads with clearly defined phase boundaries. The threads were wound up on the rod, the solution poured out, and to the test tube was added 5 ml of substrate medium containing various quantities of Mg or Ca chlorides. The samples were incubated at 37° for 1 hour. At the end of incubation 1 ml of a 70% solution of trichloroacetic acid was added to the sample, and after filtration and dilution to a definite volume, we determined the inorganic phosphate in the clear solution by the method of Fiske and Subbarow. The intensity of the coloration was measured in a photoelectric colorimeter. The activity was determined by the increase in the inorganic phosphate of the medium and expressed as μg of phosphorus liberated per hour per 1 mg N.

For control, we verified the quantity of hydrolyzed phosphate of the nucleoprotein under the same conditions. With fresh preparations the quantity of hydrolyzed phosphorus was an insignificant amount. The glycerophosphate solution was prepared immediately before beginning the experiment; under these conditions in the control experiments, phosphorus was almost undetectable in the substrate.

Determination of the Contractile Properties of Desoxyribonucleoproteins

As a test for the determination of the contractile properties we took the relative degree of relaxation of the oriented threads of DNP under definite conditions of temperature and composition of the medium.

Oriented nucleoprotein structures were produced by the method recommended by Spitkovsky [18], based on the spreading of a drop of DNP solution under the influence of gravity. A drop of a solution of DNP with a volume of 0.01 ml was carefully brought to the surface of the medium, which was placed in a graduated cylinder. As the drop fell freely within the medium, a thread was formed, 0.25 to 0.3 mm in diameter and of uniform thickness, the upper end of which was held by the force of surface tension while the lower end remained free. While the drop was falling, the nucleoprotein molecule was being oriented into an axis of flow and forming a gelatinous thread with a clearly defined phase boundary. The length of the thread with a drop of this size reached 25 to 30 mm. The degree of spontaneous relaxation which began as a result of formation of the thread was measured after 5 minutes. The relative amount of relaxation represented the ratio of the decrease in length of the thread to its original length.

The experiments were carried out at a temperature within the medium of 10 to 11°.

EXPERIMENTAL

1. Contractile Properties and Phosphatase Activity of Preparations of Desoxyribonucleoprotein Under Various Conditions of Mineral Composition of the Medium

Data have recently been published stating that nucleoprotein, like myosin, has the property of contraction.

This property was observed by Zbarsky [19] in relation to protein complexes of cell nuclei, and by Vorobyev and Shapot [20] in relation to artificially produced complexes of nucleoproteins. Spitkovsky [18], studying the contractile properties of DNP obtained from calf thymus, observed changes in these properties under various conditions of temperature and mineral composition of the medium; he established that the contractility of the threads (relative relaxation) decreases in the presence of Ca salts (0.5%), while in the presence of Mg salts, it increases. There are many data in the literature regarding the action of Mg ions on phosphatase. It has been shown that the phosphatase of the liver, kidney, intestinal epithelium, brain [21, 22] and thyroid gland [23] are activated by magnesium. The inactivating effect of Ca ions on phosphatase has also been established [24].

We studied the changes in the contractile properties and phosphatase activity of desoxyribonucleoproteins under identical conditions of concentration of Mg and Ca ions in the medium. Since the property of contractility may be related only to the nucleoprotein complex and to the proteins more or less intimately bound to it, a comparison of the changes in these properties with enzyme activity could make possible resolution of the question of the connection between the nucleoprotein and the enzyme.

Changes in the contractile properties of DNP threads were studied in the medium of medinal buffer (pH 9.4) both without and with glycerophosphate in concentrations used in experiments in the determination of enzyme activity.*

Data obtained from experiments in the study of changes in enzyme activity and in the contractile property of desoxyribonucleoprotein from calf thymus as a function of the concentration of Ca and Mg ions is shown in Figs. 1, 2 and 3.

From the results obtained it is seen that an increase in the concentration of the Ca salt in the substrate medium inhibits the alkaline phosphatase activity of DNP: at a concentration of 0.003 M, by an average of 30% and at a concentration of 0.005 M, by 66%; an increase in the concentration of Mg salts in the medium activates the enzyme and a definite maximum of activation of the enzyme (about two times) is observed at a Mg concen-

* Measurement of the relative relaxation of the DNP threads was done by N. P. Golubeva.

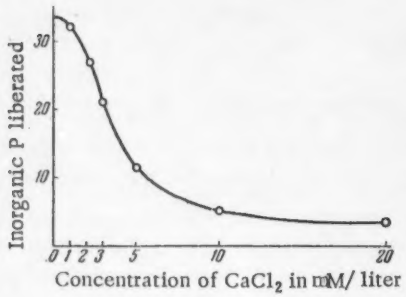

Fig. 1. Phosphatase activity of DNP as a function of the concentration of Ca in the medium.

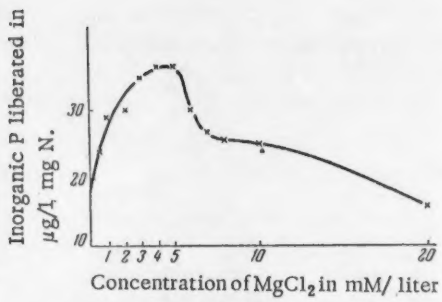

Fig. 2. Phosphatase activity of DNP as a function of the concentration of Mg in the medium.

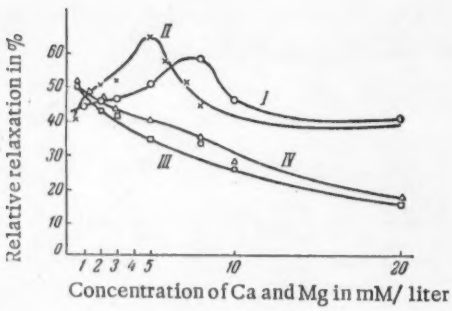

Fig. 3. The contractility of DNP as a function of the concentration of Ca and Mg in the medium. I) In the presence of Mg without glycerophosphate; II) the same, with glycerophosphate; III) in the presence of Ca without glycerophosphate: IV) the same, with glycerophosphate.

tration of 0.005 M, but further increase of the Mg ion concentration results in decreased degree of activation of the enzyme.

Similar results were obtained in studying the contractility of DNP threads. When Mg concentration in the medium was increased, contractility of the threads increased; their degree of relative relaxation increased to a definite limit after which it began to decline. When Ca concentration in the medium was increased, the degree of contractility of the thread gradually decreased. Curves of the change in enzyme activity and relative relaxation are very similar in shape. Optimal zones of concentration of Mg also coincide, corresponding to maximal enzyme activity and maximal ability of the threads to contract in a medium containing the substrate for this enzyme, glycerophosphate. Data obtained show a relation between the contractile properties of DNP and its phosphatase activity and consequently also the presence of a more or less close connection between the protein, which has enzyme functions, and the structure of the nucleoprotein complex.

It is also interesting to note that the contractile properties of the threads of DNP change under the influence of the glycerophosphate: the elasticity of a thread increases somewhat, and the optimal zone of concentration of Mg is shifted. It may be assumed that a change in these properties is also caused by interaction between the complex (enzyme) and the substrate.

In the experiments which we carried out we obtained quite definite data with regard to the inactivating action of Ca salts on the alkaline phosphatase activity of desoxyribonucleoproteins.

From the point of view of these results it becomes comprehensible why, in Stern's work [3] the desoxyribonucleoprotein developed less phosphomonoesterase activity than in the work of Presnov [1]. Stern, studying the activity of DNP with respect to combinations with various types of phosphorus linkages, used calcium salts as an activator in all his experiments. The use of calcium by this author was motivated by the results of his previous studies in which it was established that nucleoproteins, being localized in the chromosomes, show enzymatic activity with respect to compounds containing phosphorus linkages rich in energy. Having in mind the possibility of discovering similar enzymatic activity also in preparations of isolated DNP, Stern used for his experiments Ca salts which are known to be activators for ATPase. However, the use of Ca for the activation of phosphatase was erroneous.

TABLE 1

Reactivation of Phosphatase as a Function of the Degree of Inactivation of the Enzyme by Calcium

Molar concentration of Ca salt solution used for inactivation	Phosphatase activity in μg of liberated phosphorus per 1 mg N		Reactivation compared to control %
	Of the inactivated enzyme	Reactivated by magnesium (0.005 M)	
Control	12.0	24.5	100
0.001	11.5	24.0	98
0.005	9.6	20.8	85
0.01	6.4	18.6	75.6
0.05	4.2	13.8	56.3

We also studied the reversibility of the inactivating effect of calcium salts as a function of the concentration of the salts used for inactivation.

Preparations of DNP in the form of gelatinous threads (obtained by the method described above) were incubated at 37° in a substrate medium containing various quantities of Ca salts for one hour, after which the medium was poured off, the DNP threads were washed several times with fresh buffer solution not containing salts, and then treated with 5 ml of a medium containing magnesium salts at a concentration of 0.005 M (corresponding to the maximal activating effect). After incubation for 1 hour the phosphatase activity of the preparation was determined. Threads of DNP not inactivated by calcium served as controls.

It is seen from the data of Table 1 that the calcium inactivation of phosphatase is reversible. The degree of reversibility of the enzyme action depends on the concentration of calcium used for the inactivation. For low concentrations (0.001 M) the enzyme activity almost regains its original value, but with high concentrations of Ca (0.05 M) the enzyme is only 56% reactivated.

The observed specific action of the ions, as well as the reversibility of this action, may evidently be of importance in the body as one of the possible mechanisms for regulating the rate of catalytic reactions in balanced metabolism.

2. Dependence of Phosphatase Activity on the Structural State of Desoxyribonucleoprotein

The phosphatase activity of desoxyribonucleoprotein isolated from calf thymus and from the liver of large horned cattle was studied under various physical conditions of the complex: in solution, in a state of oriented, gelatinous threads, and in the form of threads dispersed under the influence of sonic activity. All these changes are characterized by various molecular-structural changes of the complex: by changes in configuration (coagulation) of the molecules in salt solution [25], by regulation of the elongated structure in gelatinous, oriented threads, and by scattering of the threads in highly dispersed sols. The explanation of the dependence of the phosphatase activity of the complex on one or another structural state of DNP could be of significance in the plan of this study to explain the relation of phosphatase to nucleoprotein.

The study of the enzymatic activity in solution appeared to be somewhat difficult since DNP, as is known, dissolves at either very low or very high ionic concentrations of the medium.

We investigated solutions of DNP in 1 M NaCl since, to obtain aqueous solutions, protracted dialysis was necessary; but in this process, changes in enzymatic activity took place. As a result of the difference in the conditions of ionic concentration of the medium in the experiments, we studied separately the effect of increasing the concentration of NaCl on the enzyme activity of DNP. The gelatinous threads of DNP were produced by the method described above.

To obtain highly dispersed sols, DNP threads were subjected to the action of high-frequency sound (8000 c.p.s.) on a sound magnetostriction apparatus for 5 minutes at 200 to 210 volts and a temperature of 2 to 4°.*

* Sound treatment of DNP preparations was carried out in the S. L. Pupko Laboratory of Electronic Microscopy, for which we here express our deep gratitude.

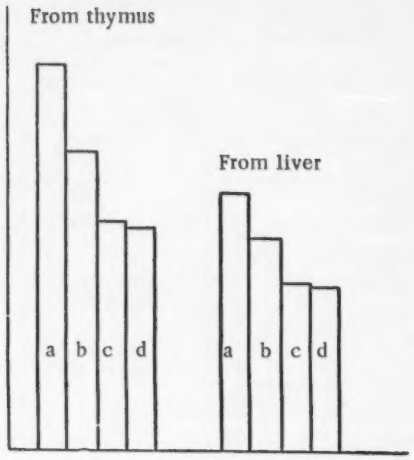

Fig. 4. Enzymatic activity as a function of the structural condition of DNP. a) Sol (dispersed thread); b) thread; c) solution; d) sound-treated solution.

The nature of the effect on desoxyribonucleoprotein of magnetostrictive sonic activity is not described in the literature. There are data concerning the depolymerizing action of zonal vibration on DNA [26] and some communications on the nature of the effect of high-frequency sound (9000 c.p.s) on tobacco mosaic virus [27, 28]. According to these data, sonic treatment produces scattering of the long, core-like structures of the virus into shorter filaments, but this scattering is not accompanied by chemical changes of the molecule. An increase in exposure of the virus to the action of sound leads to the loss of its biological activity.

The conditions of our experiment used a period of sound treatment in which was brought about the effect of apparent dispersal of the threads without a change in the enzymatic activity of the complex. The latter was verified in special control experiments in which we studied the effect of sound on the enzymatic activity present in nucleoprotein solutions.

The activity of alkaline phosphatase was determined at pH 9.4 in medinal buffer containing glycerophosphate and activating Mg ions. A DNP sol was produced by sonic treatment of threads in a substrate medium of the same composition. DNP solutions were obtained by disolving the threads in the same substrate medium containing 1 M NaCl.

For each experiment in the study of enzymatic activity we carried out separate controls to determine how much inorganic phosphorus had been liberated as a result of hydrolysis of DNP in the corresponding structural state (gel, sol, or solution).

TABLE 2

Effect of NaCl Concentration on the Activity of DNP as Alkaline Phosphatase (Phosphatase activity in μg of liberated P per 1 mg of N)

NaCl concentration in substrate medium (in moles/liter)	Desoxyribonucleoprotein	
	From thymus	From liver
1.0	40.0	22.7
0.75	40.0	22.7
0.6	40.0	23.0
0.4*	41.7*	27.5
0.3	41.7	27.5
0.15	51.0	32.7
0.05	51.0	–

* Beginning of formation of structures.

The experimental data contained in Fig. 4 and Table 2 show that the phosphatase activity of DNP in the form of a sol is higher than as a gel, and the latter is higher than the solution. These results are difficult to reconcile with the hypothesis that the phosphatase activity of DNP is related to the water-soluble enzyme which is adsorbed on the surface of the complex, since in this case one would have expected an increase in activity in the form of a solution, i.e. when there is maximal development of the active surface of the enzyme. Rather it may be assumed that the activity of the enzyme is related to the structural state of the protein-nuclein complex; to the presence of a certain regulation; to a true orientation which forms it into a protein molecule or micelle.

Thus, in the state of a structured thread there is seen an increase in enzyme activity of DNP in comparison with the solution, regardless of the considerably less favorable conditions, in this case, for the exchange between the gelatinous threads of nucleoprotein and the substrate medium. But when the threads are dispersed under the influence of sound, the enzyme activity increases, evidently as a consequence of the development in this process of an active surface of the enzyme, the elementary structure of which, responsible for the enzymatic function, is not disturbed by this action.

As shown by control experiments in the sonic treatment of DNP solutions, the action of sound in this application of sonic treatment does not appear to affect the enzymatic activity. According to previous data, increasing the intensity of the sound beam leads to supression of the phosphatase activity of DNP. It is possible that this case is like those cited above [27, 28] in which it was shown that the scattering of a virus above a certain limit led to the loss of its biological activity. The study of the intensity of action of sound in connection with a change in structure and biological activity of DNP could serve as the subject of a special investigation.

Experiments in the study of the dependence of enzymatic activity of DNP on the concentration of NaCl in the medium also showed that the phosphatase activity of the complex is related to the structure of the DNP. Thus, within the limits of NaCl concentration from 0.4 to 1 M, i.e. when the DNP is in solution, the enzymatic activity does not change, but at an NaCl concentration lower than 0.4, when a phase-delimited structure is formed, the enzymatic activity of the complex increases.

DISCUSSION

The experimental data obtained show the existence of a direct relation between phosphomonoesterase activity and the structural state of the protein-nuclein complex. The presence of such a relation was first noticed by us in the determination of the enzymatic activity of nucleoprotein solutions which had been stored for some time. It appeared that on prolonged storage in the cold (more than 3 weeks), DNP solutions (in 1 M NaCl) gradually lost the capability of forming elastic structures on dilution of the solution to a concentration of 0.14 M NaCl, and simultaneously the catalytic activity of the complex was lost. The loss of enzymatic activity, like the loss of the ability to form a structure, may be considered as a symptom of beginning "denaturation" of the nucleoprotein.

The relation between the catalytic activity of the complex and its structural state is shown by experiments in determining the comparative activity in various physical states: here it appeared that the protein-nucleic acid complex is more active when it is in a structure with oriented fibers than when it is in solution. When insoluble DNP structures are dispersed, increasing the active surface, the catalytic activity of the complex is also increased.

The study of the enzymatic activity as a function of the concentration of NaCl also showed that an increase in activity also corresponds to a condition in which structures are formed.

It was shown by special experiments that the enzymatic activity of the complex is related to the contractile properties of the nucleoprotein thread. Factors which lower (Ca) or raise (Mg) contractility produce corresponding changes in the enzymatic activity, while the nature of the change of this activity coincides with the optimal zones of concentration of Mg corresponding to the maximal development of these properties in the medium containing the enzyme's substrate.

The property of contraction reflects certain regularities of pattern of structure of the protein or system of proteins; it reflects their ability to change internal structure or to change the configuration of the molecule (in the sense of their ability to twist, i.e. by becoming shorter or longer), as determined by the molecular-physical and mechanical properties of the system.

The presence of an interrelation (which we established experimentally) between the enzymatic activity and the structural state of the DNP as well as the contractile properties of nucleoproteins, may serve as a basis for a hypothesis regarding the presence of a more or less intimate connection between the protein-enzyme and the protein-nucleic acid complex. It may be assumed that the protein, having the property of an enzyme, is one of the components of a system of proteins representing the nucleoprotein complex.

The relation between the phosphatase activity and the contractile property of the fibrillary protein-nucleic acid complex of cell nuclei may have a certain biological significance.

The works of Engelgardt [29-32] and of Szent-Gyorgyi are now well known. The former showed by the example of actomyosin the capability of a protein complex to catalyze the reaction supplying energy (splitting of ATP) which is combined with the capability of transforming this energy into a definite form of biological work – the contraction of muscle fiber.

It was in connection with the observed contractility of nuclear proteins that Zbarsky [19] expressed the hypothesis that this phenomenon is related to the mechanism of mitosis, to the formation of the spindle threads

and of the metaphase chromosomes. It is known that the chromosomes contract to a noticeable degree during mitosis. The data of a number of histochemical investigations have established [10, 34] that alkaline phosphatase is always contained in regions of the interrupted contraction of chromosomes. On the basis of these observations, Danielli [34] assumed that phosphatase constitutes an integral part of the contractile protein system, participating in the contraction of the chromosome. The author expresses the opinion that the enzymes isolated in vitro differ from the original phosphatase present in the cell, the enzymatic function of which is considerably broader.

Actually, a number of data go to show that phosphatase is a protein with polyvalent catalytic functions [30]. It is now well known that the function of phosphatase is not limited to the ability to catalyze the hydrolysis of monophosphate ester bonds. Levyant [35] obtained a series of data which served as a basis for the hypothesis that phosphatase and phosphoamidase are identical. The author postulates that both these enzyme functions belong to one protein, the different enzymatic activity of which is caused by different active groups. There are numerous data regarding the synthesizing action of phosphatase [36-39]. The work of Axelrod [38] and of Meyerhof and Green [39] is of great interest; they show that the synthesizing action of phosphatase is explained by the ability of these enzymes to catalyze transphosphorylation – the transfer of phosphate from phosphorus linkages with a high energy level to linkages with a low energy level. When phosphorus groups are transferred, energy is liberated which is expended, evidently, on the synthesis of protein.

A similar function of phosphatase agrees fully with the numerous investigations which show heightened phosphatase activity in tissues at periods related to a high rate of protein synthesis: in cell division [8, 10], in regeneration processes [40], in embryonic tissues [41], in the growth of tumors [42], and others.

In the light of this information, the data which we obtained are of interest as showing a close relation between phosphatase and nucleoproteins.

It is possible that further investigation in this direction will lead to an explanation of one of the mechanisms by which nucleoproteins take part in the metabolism associated with the synthesis of protein. Based on this there is a greater probability of the participation of nucleoproteins in the catalysis of the reactions associated with the liberation of the energy spent in the synthesis of the peptide linkage. It is also obvious that ability to catalyze the reaction connected with the liberation of energy is only one of the possible biological functions of nucleoproteins. The union of these functions with the property of the complex of contracting evidently has a definite biological significance.

SUMMARY

We studied the activating effect of magnesium salts and the inactivating effect of calcium salts on the activity of alkaline glycerophosphatase and on the contractile properties of desoxyribonculeoproteins. We established the presence of an interrelation between the catalytic activity and the contractile properties of the complex. It was also shown that the activity of the enzyme was dependent on the structural state of the nucleoprotein. The results obtained show a close relation between the protein causing phosphatase activity and nucleoprotein.

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PROTEIN METABOLISM IN EXPERIMENTAL HYPOTHYROIDISM IN THE RAT

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Many experimental studies have made reference to the great role played by the hormone of the thyroid gland in the processes of catabolism. In addition, there are certain data which make possible the assumption that the thyroid hormone is also capable of stimulating the synthesizing processes [1-3]. This latter question, regardless of its great theoretical and practical importance, has been little studied.

By study of nitrogen components of the blood of animals with experimental hypothyroidism caused by the injection of 6-methylthiouracil (MTU), Dergousova [4] noticed an increase in the level of nonprotein nitrogen in the blood of rats, while in other animals— rabbits, guinea pigs, and dogs— the non-protein nitrogen content of the blood under these conditions decreased.

These observations, together with the references of Persike [5] to the increase in the urea content in the blood of thyroidectomized rats, led us to study the rate of decomposition and synthesis of protein in various tissues of the rat under conditions of inhibition of the hormone-forming function of the thyroid gland to explain the cause of the increased content of nonprotein nitrogen in the blood of the rat and the significance of the thyroid hormone in this phenomenon.

The results expressed below show that inhibition of the thyroid secretion is accompanied by a disturbance in protein synthesis.

METHOD

The investigations were carried out on rats receiving the usual ration to which had been added 0.2 g of fish fat and 0.25 g of brewer's yeast.

The rats were made hypothyroid by the oral administration over a period of 25 to 36 days of 6-methylthiouracil (10 mg per 100 g of body weight of the animal).

Since we had at our disposal rats of various weights and sizes, in order to exclude the effect of these factors in the discussion of the data obtained, we studied simultaneously the experimental and control rats which were given the same quality and quantity of food at the same time. The rats of each pair were initially of almost the same weight and were of the same sex and litter.

How fast the protein was renewed was determined by the rate of assimilation of methionine-S^{35} into the protein of the various tissues. To this end the rats were injected subcutaneously with radioactive methionine at 10,000 impulses/gram of body weight every 24 hours until the end of the experimental period; from this moment the animals were fasted. The animals were killed by decapitation.

The radioactivity of the sulfur was determined in proteins extracted from organs and tissues by precipitation with a 10% solution of trichloroacetic acid. The protein precipitate was washed several times with 5% trichloroacetic acid, defatted by 20-hour extraction and washing with a mixture of ether and alcohol, and then with ether alone. The precipitate was dried to constant weight and the radioactivity of the sulfur determined in 10 mg of dry protein.

We judged the rate of proteolysis by the increase in nonprotein nitrogen after incubation of the tissue homogenates in acetate buffer (pH 4) for 2 hours at 38°.

RESULTS

The functional condition of the thyroid gland exerted an effect on the weight of the body and of the individual organs. Trendelenburg [6] states that the degenerative changes appearing in the internal organs after thyroidectomy lead to a decrease in their weight. Our studies showed that treating rats with MTU for 25 to 36 days led in all cases to a decrease in body weight in adult rats and to a smaller increase in body weight in growing rats in comparison with control animals.

TABLE 1

Weight of Animals and the Absolute Weight of the Organs of Control and MTU-treated Animals

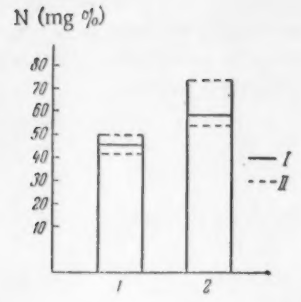
Experiment No.	Length of Observation, Days	Controls						Experimental					
		Weight of animals		Weight of organs				Weight of animals		Weight of organs			
		Initial	Final	Thyroid gland in mg	Liver in g	Kidneys in g	Spleen in g	Initial	Final	Thyroid gland in mg	Liver in g	Kidneys in g	Spleen in g
1	30	180	202	19	5.51	1.61	0.69	202	180	35	5.52	1.30	0.39
2	30	221	220	—	7.10	1.60	0.55	221	200	—	6.40	1.80	0.49
3	30	230	221	—	6.50	1 80	0.85	250	231	—	6.40	1.65	0.45
4	30	230	240	—	7 05	1.97	0.65	230	202	—	6.05	1.75	0.49
5	30	225	230	—	6 51	1.76	0.55	240	220	—	6.30	1.70	0.50
6	30	220	220	—	5.82	1 81	0.66	211	179	—	5.60	1.70	0.44
7	30	130	175	25	5.90	—	0.90	130	120	34	5.20	1.20	0 80
8	36	110	165	19	6 10	1.52	0.98	110	115	75	4.10	0.85	0.30
9	26	135	175	11	6.80	1.40	0.77	125	125	34	5.30	1.83	0.32
10	30	105	160	13	8.27	1.50	1.10	100	105	37	6 15	1.15	0.65
11	31	105	155	14	7.05	1.30	0.85	110	125	45	6.80	1.40	0.55
12	25	140	160	14	5.50	1.10	0.70	130	120	34	5.21	1.20	0.80
13	26	85	155	—	6.50	1.21	1.00	85	100	26	4 92	0.90	0.47
14	30	80	120	—	5.20	1,22	0.95	80	85	27	4.30	0.70	0.30

From a comparison of the weights of the internal organs of the control and experimental rats of each pair, a certain decrease was seen in the absolute weights of the liver and kidney and a considerable decrease in weight of the spleen of the experimental rats. Experiment 8 (Table 1) is conspicuous. In the presence of a considerable hypertrophy of the thyroid gland (which reflects the degree of hypothyroidism of the animal) a very substantial decrease is observed in the weight of the liver, kidney and especially of the spleen. The weight of the latter is three times less than the weight of the organ in the paired normal animal. This decrease in the weight of the liver and kidney on administering MTU is very similar to that observed by Persike [7] in thyroidectomized rats.

Comparison of these data gives us a basis for considering that the delay in growth and development of the MTU-treated rats arises as a result of their hypothyroid condition. The fact that thyroidectomy and chemical inhibition of the hormonogenic function of the thyroid lead to inhibition of growth of the rat, and to a decrease in the body weight of adult rats and in the weight of their internal organs, shows that disturbing the normal secretory function of the thyroid activates processes of catabolism. This hypothesis is supported by data from the literature [5, 7] and also by the observations of Dergousova who showed that the level of residual nitrogen in the blood of rats which had received MTU for 25 to 36 days was higher than in control animals.

A study of the blood of 25 experimental rats and the same number of control animals showed that the content of residual nitrogen in the blood of the latter varied between limits of 42 to 49 mg per cent (average, 46.6 mg per cent), while in experimental hypothyroidism the limits were from 56 to 73 mg per cent (average, 59.8 mg per cent).

Extremely close values for blood residual nitrogen were obtained by Leathem [8] in experiments on rats which had been fed thiourea as goitrogen for 22 days.



Blood residual nitrogen: 1) normal; 2) hypothyroid; I) average; II) minimum and maximum.

In order to find an answer to the question of the cause of the increase in the blood residual nitrogen level when thyroid function is inhibited by a goitrogen, experiments were conducted in the determination of the rate of the proteolytic processes in various tissues.

A comparison of the increase in nonprotein nitrogen after incubation of homogenates of organs and tissues of normal rats and those having received MTU shows more autolysis in the tissues of experimental rats, in almost all cases (Table 2).

A higher rate of decomposition of protein takes place on incubation of the spleen of experimental animals. On the average, the increase in nonprotein nitrogen after autolysis of the spleen is higher by 51.4% than the values which are characteristic for normal organs; this is followed by the liver (+ 34.3%) and the kidney (+ 15.8%). The difference is not as clear between the rates of decomposition of protein of the muscles of experimental and control rats; we must not look for essential differences in the increase in nonprotein nitrogen after autolysis in single cases, for sometimes the rate of the proteolytic processes in the muscles of experimental animals is even lower than in the muscles of normal animals (Experiments 3 and 9). On the average, the activity of the proteolytic process of the muscles of animals which have received MTU is higher than the normal by 11.2% (Table 2).

TABLE 2

Rate of Proteolysis in the Tissues of Normal and Hypothyroid Animals (N = normal, E = experimental)

Experiment No.	Increase in nonprotein nitrogen after incubation, mg %							
	liver		kidneys		muscles		spleen	
	N	E	N	E	N	E	N	E
1	646	709	852	911	176	183	769	852
2	612	753	812	968	167	183	811	1236
3	648	705	808	911	175	198	667	1193
4	697	754	676	787	153	116	792	1258
5	590	903	647	852	230	317	760	1142
6	592	666	649	692	124	194	706	1112
7	417	912	707	925	128	164	735	953
8	734	920	802	913	223	278	963	1577
9	537	957	652	609	233	269	632	946
10	519	937	643	862	184	127	1092	1537
11	660	861	807	892	140	184	723	965
12	640	750	652	952	159	207	769	1111
13	643	805	870	971	225	178	713	1027
14	599	822	836	823	—	—	865	1541
							834	1508
Average	609	818	744	862	178	198	789	1195
Change in %	+34.3		+15.8		+11.2		+51.4	

Thus we see that inhibition of the thyroid secretory function causes an increase in activity of proteolytic processes in all the organs and tissues studied, and especially in the spleen, by which it is possible to explain a considerable loss in weight of the latter in experimental hypothyroidism.

The data in the literature provide a basis for considering that the rate of resynthesis of protein may be estimated by the rapidity with which S^{35}-labeled methionine which is introduced into the body is assimilated into the protein of the tissue.

In Table 3 are shown the results of studies of the rapidity of the assimilation of S^{35}-methionine into the liver, kidney, spleen and muscles of normal and MTU-treated animals. In almost every case the assimilation of the amino acid into the protein of organs and tissues of hypothyroid animals is lower than in normal animals. On the average the rate of assimilation of methionine into the protein molecules decreases in the liver by 20.7%, in the kidneys by 27.4%, and in the spleen by 25.3%. Less distinct results were obtained in the muscles. However, in most cases it is possible to distinguish a certain decrease in the rate of renewal of their protein.

TABLE 3

Rate of Assimilation of Methionine Into Proteins of Organs in Experimental Hypothyroidism (N = normal; E = experimental)

	Number of impulses/ min. per 10 mg protein					
	liver		kidneys		spleen	
	N	E	N	E	N	E
	483	280	998	535	390	190
	500	380	856	584	338	338
	360	285	998	535	331	338
	323	295	527	458	238	195
	464	305	840	619	203	155
	538	470	840	621	224	166
	534	428	701	600	366	110
	340	306	982	499	321	276
	336	255	429	278	244	198
	572	526	935	644	329	269
	—	—	—	439	329	230
Average	445	353	727	528	301	225
Change in %	—20.7		—27.4		—25.3	

These data provide a basis for considering that along with an increase in the decomposition of protein in the tissues of the MTU-treated rat, decreased synthesis of protein also takes place.

DISCUSSION OF RESULTS

Our investigations showed that suppression of the secretory activity of the thyroid by treatment with 6-methylthiouracil is accompanied by an increase in the proteolytic activity of the enzymes in the organs which we studied, and by a decrease in the rapidity with which methionine is assimilated into proteins, which shows a decrease in the rate of synthesis of the protein molecules.

An increase in the level of the blood residual nitrogen is caused by these changes in protein metabolism; this increase is seen in chemical suppression of the hormonogenic function of the thyroid [4, 8]. In all probability these changes also cause inhibition of the growth of the rat, a decrease in weight, and a decline in weight in the internal organs both under conditions of experimental hypothyroidism and of thyroidectomy.

The thought may arise that the increase in the amount of nonprotein nitrogen in the blood of MTU-treated rats takes place as a result of lowered food intake by the experimental animals, which leads to a higher rate of decomposition of the protein of the tissue.

Opposition to this idea is expressed by the work of Persike [7], who found a higher urea content in the blood and a higher rate of excretion of it in the urine in thyroidectomized rats in comparison with control animals which had received an amount of food which was limited to correspond to the diet of thyroidectomized animals which

suffered a certain loss of appetite. We, however, were unable in general to notice a great difference in the food consumed by experimental and control animals.

A substantial difference is seen in the synthesis of proteins in hunger and in hypothyroidism. Thus, the studies of Konikova et al., [9] showed that the assimilation of sulfur-containing amino acids into the protein of organs and tissues of starving rats is considerably higher than in rats on a normal diet. As seen from our experiments, the assimilation of S^{35}-methionine into the proteins of organs decreases in hypothyroidism.

The latter circumstance provides a basis for considering that the decrease in protein synthesis is caused by a disturbance in the secretory activity of the thyroid gland, and, consequently, the thyroid hormone plays a part not only in catabolic, but also in anabolic processes. This is supported by the study of Salganik [3] showing that in the period following two-day starvation, the restoration of body weight, of the quantity of protein in the liver, and of the weight of the liver takes place more slowly in MTU-treated rats than in animals with undisturbed thyroid secretory function.

Rupp, et al., [2] mention that a shift in the action of thyroid hormone to the side of synthesis of protein, or its decomposition, depends on the dose of thyroxin. In experiments with thyroidectomized rats they discovered that small physiological doses of thyroxin decrease the nitrogen content of the urine, while large doses increase the excretion of nitrogen.

The ability of the thyroid hormone to strengthen the anabolic processes was reported by the clincial observations of Agafonov [10] and of Olevsky and Kaplansky [11]. Using small doses of thyroidin for the treatment of children of less than normal growth, they observed an increase in weight and an improvement in their general condition.

The authors state that in many cases increasing the protein content of the diet was not enough to assure normal development both in premature infants and in undersized children. The children could not use the protein sufficiently for building tissue and only the administration of small doses of thyroidin made possible growth and development of their bodies.

Thus, both the data from the literature and also our experimental observations provide a basis for considering that the thyroid hormone has a very decisive role in the synthesis of the protein molecule.

SUMMARY

Inhibition of the secretory function of the thyroid gland by 6-methylthiouracil leads to an increase in the quantity of non-protein nitrogen in the blood of the rat, which is caused by a faster rate of decomposition of protein of the tissue and a decrease in the synthesis of it.

The results of the investigation give us a basis for assuming that the thyroid hormone has a decisive role in the renewal of the proteins of the tissues.

Received April 6, 1956

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LOCALIZATION OF PROTEIN SYNTHESIS IN THE PLANT CELL

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A detailed study of the role and importance of the plastids in plant physiology has showed that they play an extremely important part in the various synthesizing functions of the plant cell [1, 2]. Studies by Sisakyan et al.,[3-5] have established that plastids are the original "depots" of the enzymes; the fundamental group of plant enzymes is intimately connected with the structure of the plastids. The enzymatic activity of the plastid depends both on the nature and physiological condition of the organism and on the character of the union of the enzymes with the protein complex of the plastids [1].

Of special importance is clarification of the question of the participation of the plastids in the synthesis of protein. Work done in this field [1, 2, 6] has shown that when plastids are incubated with glycylglycine containing C^{14} in the presence of ATP, succinate, and fumarate, the dipeptide is assimilated into the protein of the plastid at a fairly high rate. In connection with the data regarding the important role of the plastids in the synthesizing functions of plant cells, and, in particular, the role of plastids in protein synthesis, it is of interest to study comparatively the rate of synthesis of proteins in the plastids and cytoplasm of the leaves of plants. We carried out this study of the synthesis of proteins by determining the quantity of S^{35} assimilated into the proteins of the plastids and cytoplasm in the vacuum infiltration of bean leaves by S^{35}-methionine and $Na_2S^{35}O_4$.

EXPERIMENTAL

Leaves of the two weeks' growth of the "Triumf-lushchilny"* variety of the bean plant served as the subject of the study. Samples of the leaves were infiltrated with a solution of S^{35}-methionine or $Na_2S^{35}O_4$. The specific activity of the labeled methionine amounted to 223 μc/ mg and for the infiltration we used a solution containing 1.12 μc of S^{35}-methionine per ml. The activity of the sodium sulfate, which was used at a concentration of 0.01 M, amounted to 0.92 μc/ ml. Infiltration of the leaf samples was carried out in a moist chamber with luminescent illumination of an intensity of about 3000 lux and at a temperature of 23 to 25°. Plastids were extracted by Vecher's method [7]. After appropriate exposure the leaves were frozen solid for 30 to 40 minutes and then carefully ground in a mortar with an isotonic solution of 0.35 M glucose, and centrifuged to remove the starch, cellular tissue, intact cells, etc. for four minutes at 2500 r.p.m. The precipitate was discarded and the centrifugate was used for analysis.

Separation of plastids from the cytoplasm was carried out in a supercentrifuge without interruption at 19,000 to 20,000 r.p.m. The liquid was produced at a rate of about 50 ml/ min. The plastid fraction was collected from the film on the rotor, carefully mixed with water, and the plastid protein precipitated by 5% trichloroacetic acid. The cytoplasm protein was also precipitated by the addition of trichloroacetic acid to final concentration of 5%. The protein was filtered off and washed free of soluble nitrogenous and sulfur-containing compounds by 2% trichloroacetic acid until the disappearance of the radioactivity in the washings.

Combustion of the protein precipitate and subsequent determination of the sulfur was carried out by Benedict's method [8]. The precipitate was covered in the crucible with 10 ml of Benedict's solution (20 g $Cu(NO_3)_2$ and 5 g $KClO_3$ in 100 ml of water), dried, and kept at a dark red heat for 2 hours. The roasted precipitate was

* Literally: "Shelling triumph" - Translator.

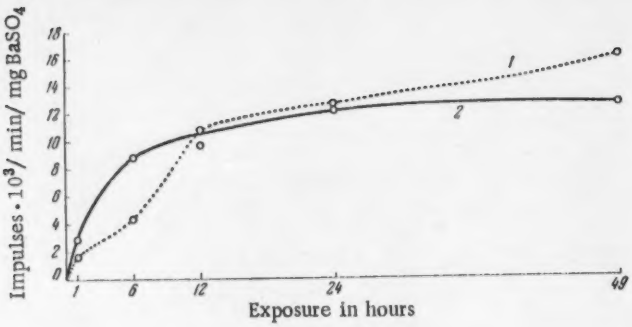

Fig. 1. Assimilation of S^{35}-methionine into protein of the plastids and cytoplasm of bean leaves: 1) Protein of the cytoplasm; 2) protein of the plastids.

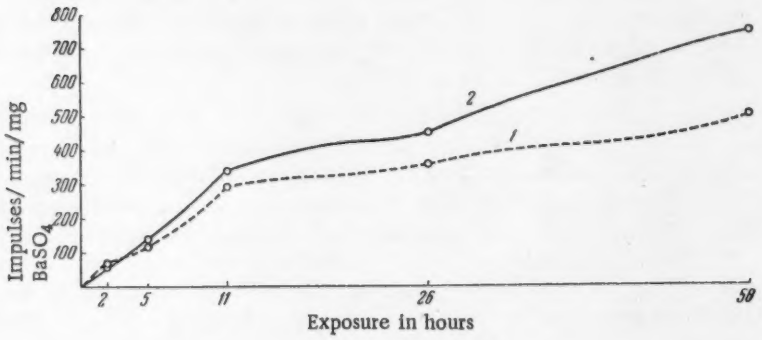

Fig. 2. Assimilation of S^{35} into the proteins of plastids and of cytoplasm of bean leaves on infiltration with $Na_2S^{35}O_4 \cdot 10H_2O$: 1) Protein of the cytoplasm; 2) protein of the plastids.

dissolved in 10% HCl and the sulfur precipitated by barium chloride as $BaSO_4$. Samples of $BaSO_4$ were prepared by Larson's method [9]. Determination of the activity of the $BaSO_4$ precipitate was carried out in brass cups with an area of 0.6 cm^2 using a BFL-25 end-window counter with a window thickness of 1.2 mg/ cm^2.

The data shown in Fig. 1 are averages of two parallel batches. The results obtained show that within an hour after infiltration of labeled methionine into the bean leaves, a very considerable quantity of S^{35} was discovered in the proteins of the plastids and cytoplasm, which shows the high rate of inclusion of methionine into the protein. From Fig. 1 it is seen that in the first hour after infiltration the rate of assimilation of S^{35}-methionine into the protein of the plastids exceeded by almost two times the rate of its assimilation into the plasma proteins. After 6 hours the quantity of S^{35}-methionine assimilated into the plastid protein exceeded more than twice the amount assimilated into the protein of the cytoplasm. On prolonged exposure (for 12 and 24 hours after infiltration) the quantity of methionine assimilated into the plastid and cytoplasm protein was almost the same, while after 49 hours the quantity of labeled methionine in the cytoplasmic protein exceeded the quantity of it in the plastid protein. Thus, the synthesis of protein at first takes place at a faster rate in the plastids (for the first 6 hours), and only after 12 to 49 hours following infiltration is a higher rate of assimilation of methionine into the cytoplasmic protein observed.

The data of Fig. 1 also show that for exposures of more than 12 to 24 hours, the S^{35}-methionine content in the plastids does not increase. Considering the high rate of turnover in proteins [10], it must be assumed that the

absence of an increase in the specific activity of plastid protein in this case may be explained by the fact that after this period replacement of the greater part of the plastid protein takes place, so that the subsequent assimilation of S^{35}-methionine is almost fully compensated by the process of protein decomposition, i.e. by the excretion of methionine from the composition of the protein molecule. The cytoplasmic protein is evidently turned over considerably more slowly, and the turnover process in the cytoplasm is not concluded even after the period of the 49-hour exposure.

Determination of the rate of assimilation of S^{35} into the protein of the plastids and cytoplasm on infiltrating the leaves with 0.01 M $Na_2S^{35}O_4 \cdot 10H_2O$ (Fig. 2) showed that in the first 5 hours after infiltration, the quantity of S^{35} assimilated into the protein of the plastids and cytoplasm is comparatively small. On more prolonged exposure (11 to 50 hours), the S^{35} content in the protein clearly increases; in this the assimilation of S^{35} into the protein of the plastids exceeds the amount assimilated into the protein of the cytoplasm.

As distinguished from methionine, sulfates cannot be used directly for the synthesis of protein in plants; they must first be reduced and then formed into sulfur-containing nonprotein organic compounds. In this case it is evident that the rate of assimilation of sulfur into protein will be limited by the rate of reduction of the sulfates. However, even in infiltration by sulfates, the rate of assimilation of S^{35} into the proteins of plastids was higher than into the proteins of the cytoplasm. This is evidently explained not only by the higher rate of assimilation of sulfur-containing amino acids into protein, by also by the fact that the reduction of sulfates and the synthesis of amino acids takes place more rapidly in the plastids than in the cytoplasm. Thus, on the basis of the data obtained, we assume that the reduction of sulfates and the rate of assimilation of methionine into the proteins takes place at a faster rate in the plastids than in the cytoplasm; the turnover of proteins in the plastids also takes place at a faster rate in comparison with the proteins of the cytoplasm.

SUMMARY

In vacuum infiltation of bean leaves with S^{35}-methionine and $Na_2S^{35}O_4$, the rate of assimilation of S^{35} into the proteins of the plastids and the cytoplasm was determined. It was shown that 1 to 6 hours after infiltration, the rate of assimilation of labeled methionine into the protein of plastids was more than twice as fast as the rate of its assimilation into the protein of cytoplasm. On exposure of more than 12 to 24 hours, no increase in the specific activity of the proteins of the plastids takes place. In infiltration by $Na_2S^{35}O_4$, the rate of assimilation of S^{35} into the protein of the plastids also exceeds the rate of assimilation of sulfur into the protein of the cytoplasm. It is suggested that the reduction of sulfates and the synthesis of sulfur-containing amino acids take place at a faster rate in plastids than in cytoplasm.

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CHANGES IN SERUM ALBUMIN ON IRRADIATION BY GAMMA RAYS IN VITRO AND IN VIVO

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The irradiation of serum albumin by gamma rays outside the body, as we showed in an earlier paper [1], produces in protein structure such changes as bring about quicker and deeper digestion by pepsin.

We considered that the cause of such increased digestibility consisted of changes in the course of denaturation beginning in the structure of the protein molecule after irradiation and allowing the hydrolytic enzymes to effect splitting of the peptide linkages with a smaller expenditure of energy. Volkova and Pasynsky [2] showed that exposure of human serum albumin and horse globulin to high-intensity x-rays did not produce any noticeable splitting of the peptide bonds. According to them, the fundamental importance of the changes in the protein molecules is not the direct changes in a small part of the molecules, but the structural and denaturational changes of the affected molecules. Fricke noticed a decrease in the energy of activation of denaturation of egg albumin after its exposure to x-rays, which certainly is related to changes within the protein of the same nature as observed by Pasynsky and by us.

In the present work, using some changes of method, we have confirmed our previous observations and discovered that the serum albumin of experimental animals which have had a source of gamma rays introduced into their bodies is subjected to the same structural changes. The radioisotope of iron, Fe^{59}, when administered with the food, is deposited in the liver of the animal, accumulates there and acts on the serum albumin the moment the latter is produced in the liver, or affects the protein which is already synthesized. Since time is required for the excretion of serum albumin from the cells into the blood stream (Khesin [4]), in the second event the already prepared protein is subjected to the same exposure to gamma radiation as we observed in vitro [1]. It is less probable that the serum albumin already in the blood might be subjected to gamma irradiation, since the quantity of iron in the serum is very small [5, 6] and the Fe^{59} incorporated into the hemoglobin at the time of the experiment can hardly be much. While renewal of the erythrocytes takes place every 110-120 days, changes in the serum albumin begin from the first few days after the Fe^{59} is introduced into the body.

The isotopic iron introduced into the body is not without consequences for the body as a whole, and especially for the liver. This is shown by the high readings in the thymol-veronal test and also in the distinct changes in the protein picture of the blood serum, which we estimated by changes in its electrophorogram. The observed relative increase in the β-globulin fraction is evidently an answering reaction to the radiation damage. The normal picture of the protein composition of the blood serum is re-established only many weeks after stopping the intake of Fe^{59}.

EXPERIMENTAL

In experiments in vitro we used 2 and 3% solutions of pure human serum albumin (prepared by the Blood Transfusion Institute in Moscow), or human blood serum dialyzed against water. The protein solution was poured into a small penicillin flask and into it was immersed a 5 mm test tube containing 1 ml of a solution of ferric chloride containing Fe^{59}.

Conductometric titration of 0.1 ml of the protein solution (pH about 6) with 0.01 N HCl was carried out as described earlier by us [7]. Titrating in aqueous solution we discovered on an average 20 equivalent titrated

Quantity of Tyrosine in the Aqueous ("Bound") and Alkaline ("Free") Eluates as a Function of the Duration of Peptolysis of Non-irradiated and Irradiated Human Serum Albumin
Irradiation, 224 μc Fe^{59}; Peptolysis at 37°

Duration of peptolysis in hours	Nonirradiated albumin		Irradiated albumin (time in hours)					
			14		28		68	
	Tyrosine, %		Tyrosine in % in the eluate					
	H_2O	NaOH	H_2O	NaOH	H_2O	NaOH	H_2O	NaOH
0	4.70	0						
0 25	4.45	0.22	4.36	0.50	0.58	4.10	0.11	4.65
1.5	4.23	0.33	3 72	1.05	0.26	4.43	0.11	4.64
2 5	3.78	0.77	2.89	1 61	0,20	4.51	0,11	4.64
4 0	3.66	0.80	2.39	2.00	0.20	4 50	0.11	4.66

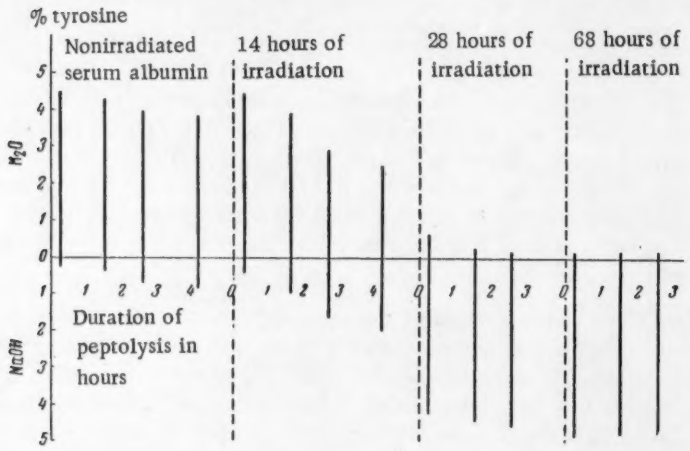

Fig. 1. Peptolysis of human serum albumin with irradiation of the protein by gamma rays of the radioisotope Fe^{59} in a test tube.

groups in one mole of protein. Titration of the solution exposed for one hour to 224 μc Fe^{59} showed a noticeable increase in the number of titrated groups – to 26. More prolonged exposure to the same solution of ferric chloride brought about an insignificant further increase in the number of titrated groups. After 17.5-hour exposure they increased to 28 per mole of protein. Evidently, as the period of exposure lengthens, there is a decrease in the number of weak bonds (essentially, those of hydrogen) which leads to an unfolding of the basic peptide chains with the liberation of a certain additional number of titrated groups.

The rate and depth of peptolysis in our work was determined by the number of molecules of tyrosine liberated in the course of digestion of serum albumin. For this purpose we added to the serum albumin solution on equal volume of a 0.1% solution of pepsin (a preparation of the Tashkent Vaccine and Serum Institute) in 0.04 N HCl. Of this mixture, 0.5 ml was at once put through a column (20× 0.4 cm) filled with anionite PEK 1 (GIPKh* Leningrad) in OH form. Subsequently, running through of 10 ml of water completely washed out the remaining insoluble protein. The remaining solution of protein with pepsin in acid medium was placed in the constant temperature oven at 37° ± 0.5°, and after various periods 0.5 ml quantities of the hydrolyzate were removed and put through the same anionite column followed by washing with water. The "bound tyrosine" remained in the aqueous eluate combined with still unsplit protein or large peptides. The free tyrosine, liberated in the process of peptide hydroly-

* State Institute of Applied Chemistry - Translator.

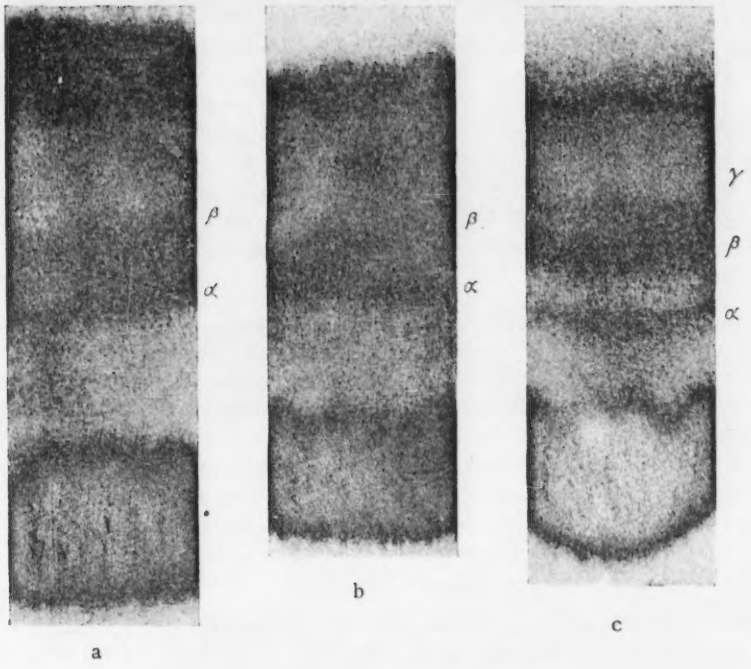

Fig. 2. Electrophorograms of blood serum: a) dog Volchok before adminstration of Fe^{59}; b) same dog on the second day after stopping adminstration of Fe^{59} (it had received a total of 4.1 mc of Fe^{59}); c) dog Bokser three months after stopping administration of Fe^{59}.

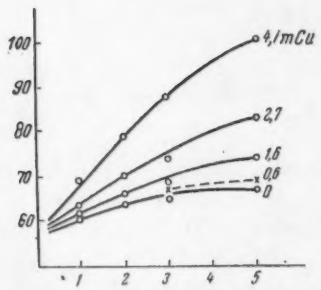

Fig. 3. Rate of Peptolysis of Serum Albumin of the Dog Volchok after Treatment with Fe^{59}.
Ordinate: quantity of titrated protein groups in equiv/mole.
Abscissa: time of peptolysis in hours.

sis, as distinguished from the peptide ("bound") tyrosine, was adsorbed on the anionite and could be quantitatively eluted with 1 M NaOH. Thus for each sample of the peptolyzate of serum albumin, both nonirradiated and irradiated, we were able to determine in the aqueous eluate the quantity of "bound" or yet unsplit tyrosine, while in the alkaline eluate we could determine the quantity of "free" tyrosine. The tyrosine was determined by the method of Zuverkalof [8].

Paper electrophoresis took place in an apparatus which we constructed of plexiglas on No. 1 chromatographic filter paper (Paper Factory No. 2, Leningrad) in 0.06 M veronal buffer at a pH of 8.6, a potential gradient of about 7 v/cm and a current of about 1 to 1.5 ma on a separate strip of paper. Processing and development with bromphenol blue took place in the usual manner corresponding to Tiselius' instructions.

From the table and from Figure 1 it is seen that the peptolysis of the nonirradiated serum albumin clearly slows down after 4 hours. After this time only 17% of the tyrosine present in the protein arrives at the stage of liberation. Of the serum albumin irradiated for 14 hours, with the same length of peptolysis, 43% of the tyrosine present is liberated; of the protein irradiated for 28 hours, only 2.5 hours of peptolysis liberates 87% of the tyrosine present. Under the action of pepsin, serum albumin which has been irradiated for 68 hours splits as if into little pieces and in a quarter of an hour we detected 98% of all the protein tyrosine in the free state.

In the work of Borisov [9] it was shown that peptolysis proceeds by a type of reaction of a fractional order (the velocity of the pepsin digestion is a function of one half the concentration of the enzyme). In our experiments, evidently, the ratio of the concentration of the substrate to the enzyme appeared to be such that the peptolysis also proceeded from the beginning according to this pattern. Further, however, it appeared that the velocity of the peptolysis was a function of the concentration of the substrate according to a first-order reaction (evidently not related to the amount of substrate remaining). The velocity constant calculated for the pepsin digestion of nonirradiated serum albumin, based on the calculation of the possible degree of splitting, was found to be 0.0014. Serum albumin irradiated for 14 hours splits more rapidly and more deeply – the velocity constant of its decomposition was 0.0027. After 28 and 68 hours of irradiation the velocity constants of decomposition were found to be of the order of 0.012 and 0.25. This acceleration is one to two orders higher than for nonirradiated protein.

The irradiated molecule of protein preserves its macrostructure, but, when it is subjected to peptide hydrolysis, decomposes into tiny fragments, which is possible only with a considerable decrease in the energy of activation of this process [3]. It is possible that the cause of such deepening and acceleration of the decomposition of peptide bonds is the considerably facilitated access to them of pepsin because of the breaking of hydrogen bonds in proportion to the rate and duration of irradiation. It is known that it is the hydrogen bonds which fundamentally keep the molecule of serum albumin, as well as of other proteins, in a stable condition. Analogous changes are undergone by the serum albumin within the animal body after treatment with Fe^{59}. In this case, in addition to the gamma radiation, there is added the considerably less intense beta radiation which we did not observe in the experiments in the test tube because it was completely absorbed by the glass walls of the test tube into which the solution of ferric chloride was poured.

We used five experimental dogs. The changes observed in their blood and general condition was exactly the same. We show experimental data for the dog "Volchok." Daily from the 5th to the 19th of October she received with her food 0.1 ml of a solution of ferric chloride containing Fe^{59} except on October 14th and 16th, when she did not receive any Fe^{59} at all, and on October 15th, when she received 0.2 ml of this solution with her food. At the beginning of the experiment 0.324 mc. were given; on the last day, October 19th, 0.263 mc. We took great care to see that the necessary quantity was entirely consumed. Samples of blood for analysis were taken on October 5th (before treatment with the radioisotope), 7th, 10th, 14th, and 22nd, i.e. after giving the animal respectively 0.643, 1.57, 2.75, and 4.11 mc. of Fe^{59}. During the entire time of the experiment Volchok, like the other dogs, did not lose any weight; the total quantity of serum protein remained the same (7.5 to 7.8%), although the relative quantities of individual fractions changed distinctly (Fig. 2). Compared with its value before the experiment, after irradiation the percentage content of β-globulin increased considerably. Only following a considerable period (6 to 8 weeks) after stopping the administration of isotopic iron did the protein picture of the blood serum return to its original condition. While the Fe^{59} was being given the thymol-veronal blood tests reached values higher than 0.10.

The rate of peptolysis of the serum albumin of the dog Volchok before and during the experiment is shown in Fig. 3. The serum albumin was freed of globulins by dialyzing the serum against distilled water. The quantity of protein was determined by the biuret test in a photoelectric colorimeter FEK M. The degree of peptolysis was determined by conductometric titration of the peptolyzate, as described by us earlier [1]. As seen from Fig. 3, the serum albumin of the dog changed little for two days following administration of the isotope. Only after 5 hours of peptolysis at 37° did a somewhat greater number of titrated groups show up as compared with the observations for the same length of peptolysis of the serum albumin of the same dog before giving the radioisotope. The course of the curve of the serum albumin in vivo is quite different from the course of the curve of the peptolysis of serum albumin not subjected to the action of the administered radioisotope.

Just as in the case of irradiation of blood albumin in vitro by gamma rays, so also in vivo the rate of peptolysis and its depth (the possibility of splitting large numbers of peptide bonds, the energy of activation of which, evidently, varied), as we saw, depends on the duration and intensity of irradiation. It is hard to say whether we are dealing with a direct change of a denaturational character of the already synthesized protein molecule, or with an incomplete synthesis of protein caused by disturbance of the work of whatever enzyme systems of the liver take part in this.

With respect to the mechanism of action of the rays of isotopic iron which accumulates in the liver and in other tissues, it is exerted fundamentally through the formation of active radicals in the water of the tissues and redoubles the oxygen present in them.

CONCLUSIONS

The radioactive isotope of iron Fe^{59}, when introduced into the bodies of dogs, accumulates in the liver and in other organs and radiates extremely intense gamma rays and the less intense beta rays. It causes changes in the structure of the serum albumin similar to those which set in when this protein is irradiated in vitro. We estimated these changes by the accelerated and deeper splitting of molecules of the irradiated protein as compared to that not irradiated.

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REVIEW

"Questions of Medical Chemistry," Vol. I, Nos. 1 to 6, 1955

(Medgiz Press, Moscow)

The journal "Questions of Medical Chemistry," having begun its appearance in 1955, replaced the separate symposia of the same name which appeared from 1949 to 1953. The replacement of these nonperiodic symposia (a total of 6 symposia appeared in 5 years) by a periodic edition was, naturally, quite expedient and answered the urgent requirements of large groups of biochemists and physicians for a special journal related to questions of medical chemistry. The great advances made in biochemistry in the past decade could not but be reflected in the development of almost all medico-biological disciplines. This point was made with great truth in the preface to the first edition of the journal being reviewed. On the basis of biochemical progress in the study of the processes of metabolism in the bodies of man, animals, and microorganisms there took place a radical reconstruction of such disciplines as general and medical microbiology, virology, immunology, endocrinology, pathologic physiology, pharmacology, chemotherapy, etc. To this it must be added that such morphological disciplines as histology and cytology become more "biochemical" with each year; a branch of these disciplines, cytochemistry, will receive the greatest emphasis in further research and development.

In connection with the ever widening introduction of biochemistry into various fields of medicine, interest is growing, not only on the part of biochemists working in clinical laboratories, but also on the part of very wide circles of clinicians, in gaining quick knowledge of the new biochemical data which may be of importance for diagnosis or for deciphering the pathogenesis of one disease or another. New journals for clinical or medical biochemistry have been appearing in various countries just for this purpose. Not long ago there appeared the first issue of an international journal for these problems, "Clinica Chimica Acta," in which a resume of each work is printed in several languages, including Russian.

In 1955 84 articles were printed altogether in the journal being reviewed, among them accounts of conventions and conferences, reviews, etc. This number should by no means be taken as sufficient. In the journal no dates are given of the arrival of the article at the editorial office, so that it is impossible to estimate the time between submission of the article and its appearance in print, but in all probability this period is more than six months. If one considers only the number of institutes and laboratories immediately engaged in problems of medical chemistry, then in order to print the most important of the papers produced not later than half a year after their submission to the editor the volume of the journal would have to be doubled, i.e. either the journal would have to become a monthly or the number of pages per issue would have to be doubled.

Increasing the size of the journal would make it possible to take care not only of the experimental works, but also systematically to publish surveys of the problems most current in medical chemistry. At present, only the journal "Progress in Contemporary Biology" publishes from time to time similar surveys, which are, incidentally, extremely necessary not only for the clinician wishing to gain knowledge of the current state of one problem or another, but also for the biochemist who does not find it possible to follow all the literature relating disturbances of metabolism in various pathological conditions of the body.

Of the 84 articles placed in the six issues of the journal in 1955, about twenty articles are related to methodological questions. Such a ratio must be considered fully correct, since it is just the introduction of new methods of investigation into the clinical laboratories which is one of the most important tasks of the journal. Many of the articles of this classification will doubtlessly interest wide groups of readers of the journal, and the methods described here will quickly be used for appropriate analyses. Among these articles are those by Vladi-

mirov and Klimov, "Chemical Methods of Determining Penicillin in the Blood and Urine"; one by Kasavina, Gorkin and Gugeshashvili on the paper electrophoresis of the protein of blood serum; one by Shlygin, Mikhlin, Fomina, and Skirko on the determination of phosphatase in the blood serum and tissues; one by Sachkov on the quantitative determination of nitrogen by isometric distillation in flasks, and a number of others.

In addition to the articles on methods, considerable space was occupied by articles dealing with the role of various disturbances of metabolism in the pathogenesis of various pathological conditions. Similar to these are articles relating to peculiarities in the composition and metabolism of malignant neoplasms (Belousova; Zbarsky and Debov; Kirsanova and Tustanovsky; Etingof and Balyasna); to disturbances of metabolism in diabetes (articles by Guly, Mikhailovska, Degtyar; Leites and Pavlov); to factors causing an increase in permeability of capillaries in inflammation (Paskhina); a study of the disturbances of liver function using the thymol-veronal test (I. A. and V. I. Oivin and Tikhonravov), etc. These papers represent profound and serious studies in very current problems of pathology, giving the reader a clear conception of the contemporary biochemical approach to the solution of complicated questions of the pathogenesis of various diseases. Naturally, not all the articles of this kind have the same value. In addition to articles which are written at a high level, one also finds those whose themes are not current and also articles whose conclusions are not convincing because of errors in methods or in the setup of the experiments. It must be noted that the number of such articles is not great and that the editors of the journal have developed sufficient care in the choice of articles to be printed.

In addition to articles related directly to questions of medical chemistry, in past issues of the journal there have been quite a few articles without a direct relation to these questions. Such articles as that by Beliko, "A Study of the Mechanism of Formation of Fibrin from Fibrinogen," or that by Brams, "Action on Muscle of Hydrogen Peroxide Formed as a Result of an Enzymatic Reaction," could have been published more expediently in a biochemical journal of general coverage, since they do not concern purely medical problems. It must be noted, however, that in many cases it is very difficult to separate articles into those with a direct relation to medicine, and those without such a relation. Further, the practice of scientific investigation has shown convincingly enough that often papers which today seem remote from urgent problems of medicine will later turn out to be very closely connected with some question of pathology and will assume great significance either in explaining the pathogenesis of various diseases or in solving questions of their diagnosis and therapy. Suffice it to say, for example, that the purely biochemical study which we carried out in the USSR on the role of adenosine triphosphate in the mechanism of muscular contraction led to the use of this substance as a therapeutic preparation in the treatment of certain forms of muscular dystrophy. Thus the editors of the journal must never be reproached for having given space to a number of papers of general biochemical importance in addition to papers dealing directly with some medical problem.

In summing up the activity of the journal in the first year of its existence, there is full justification for saying that it has had full success in dealing with the problems assigned to it and that the articles published in it on various problems of medical biochemistry are of undoubted interest to extensive groups of biochemists and clinicians. In conclusion one must once more express the wish for an increase in the number of pages and copies.

S. Ya. Kaplansky

ERRATA

Page	Reads	Should read
	Vol. 21, No. 1	
62	[13]. . J. Biol. Chem. 12, 899, 1933.	[13]. . J. Clinic. Investig. 12, 899, 1933.
	Vol. 21, No. 2	
197	80% formic acid	85% formic acid
199	Fig. 4. 14) pyrosine	Fig. 4. 14) tyrosine
201	route for the inclusion of C^{14} and $C^{14}O_2$	route for the inclusion of C^{14} from $C^{14}O_2$
236	[2] A. G. Pasynsky and R. S. Chernyak, Proc. Acad. Sci. USSR 79, 1001 (1952).	[2] A. G. Pasynksy and R. S. Chernyak, Biokhimiya 17, 198 (1952).
	Vol. 21, No. 3	
433	100-150 mC P^{32}	100-150 μC P^{32}
436	energy supplied by macroergic phosphates [5-7].	. . . phosphates [17,19].

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